This Page Is Inserted by IFW Operations and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents will not correct images, please do not report the images to the Image Problem Mailbox.



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11) EP 1 152 062 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication: 07.11.2001 Bulletin 2001/45

(51) Int Cl.7: C12Q 1/68

(21) Application number: 01109341.6

(22) Date of filing: 12.04.2001

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 18.04.2000 US 198336 P

(71) Applicant: F.HOFFMANN-LA ROCHE AG 4070 Basel (CH)

(72) Inventors:

• Smith, Edward Soh San Francisco, CA 94110 (US)

Elfstrom, Carita Maria
 San Francisco, CA 94131 (US)

 Gelfand, David Harrow Oakland, CA 94611 (US)

 Higuchi, Russell Gene Alameda, CA 94501 (US)

 Myers, Thomas William Alameda, CA 94501 (US)

 Schoenbrunner, Nancy Jeneane Moraga, CA 94556 (US)

 Wang, Alice Ming Lafayette, CA 94549 (US)

(74) Representative: Knauer, Martin, Dr. et al Roche Diagnostics GmbH, Patent Department 68298 Mannhelm (DE)

(54) High temperature reverse transcription using mutant DNA polymerases

(57) The present invention relates to improved reverse transcription methods using a modified ther-

mostable DNA polymerases, particularly in a magnesium ion buffer. These methods are particularly useful in combined reverse-transcription/amplification reactions.

Description

20

35

45

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The present invention relates to the field of molecular biology and, in particular, relates to methods for the reverse transcription and amplification of ribonucleic acid (RNA) sequences.

10 DESCRIPTION OF RELATED ART

[0002] The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA polymerases. All known reverse transcriptases require a primer to synthesize a DNA transcript from an RNA template. Historically, reverse transcriptase has been used primarily to transcribe mRNA into cDNA which can then be cloned into a vector for further manipulation.

[0003] The term "DNA polymerase" describes a class of polymerases characterized as DNA-dependent DNA polymerases. DNA polymerase show a strong discrimination against using an RNA template, as expected from their functions in vivo. Nevertheless, several laboratories have shown that some DNA polymerases are capable of in vitro reverse transcription of RNA (Karkas, 1973, Proc. Nat. Acad. Sci. USA 70:3834-3838; Gulati et al., 1974, Proc. Nat. Acad. Sci. USA 71:1035-1039; and Wittig and Wittig, 1978, Nuc. Acid Res. 5:1165-1178). Gulati et al. found that *E. coli* Pol I could be used to transcribe Qβ viral RNA using oligo(dT) 10 as a primer. Wittig and Wittig have shown that *E. coli* Pol I can be used to reverse transcribe tRNA that has been enzymatically elongated with oligo(dA). However, as Gulati et al. demonstrated, the amount of enzyme required and the small size of the cDNA product suggests that the reverse transcriptase activity of *E. coli* Pol I has little practical value.

[0004] T. aquaticus (Taq) DNA polymerase, a thermostable DNA polymerase, has been reported to inefficiently synthesize cDNA using Mg⁺² as the divalent metal ion (Jones and Foulkes, 1989, Nuc. Acids. Res. 176:8387-8388). Tse and Forget, 1990, Gene 88:293-296; and Shaffer et al., 1990, Anal. Biochem. 190:292-296, have described methods for amplifying RNA using Taq DNA polymerase and Mg⁺² ion. However, the methods are inefficient and insensitive.

[0005] Amplification of nucleic acid sequences, both RNA and DNA, is described in U.S. Patent Nos. 4,683,195; 4,683,202; and 4,965,188; each incorporated herein by reference. A preferred method, the polymerase chain reaction (PCR), typically is carried out using a thermostable DNA polymerase, such as Taq DNA polymerase, which is able to withstand the temperatures used to denature the amplified product in each cycle. PCR is now well known in the art and has been described extensively in the scientific literature. See, for example, PCR Applications, 1999, (Innis et al., eds., Academic Press, San Diego), PCR Strategies, 1995, (Innis et al., eds., Academic Press, San Diego); PCR Protocols, 1990, (Innis et al., eds., Academic Press, San Diego); and PCR Technology, 1989, (Erlich, ed., Stockton Press, New York); each incorporated herein by reference. Commercial vendors, such as PE Biosystems (Foster City, CA) market PCR reagents and publish PCR protocols. A review of amplification methods is provided in Abramson and Myers, 1993, Current Opinion in Biotechnology 4:41-47, incorporated herein by reference.

[0006] Because reverse transcription using Taq DNA polymerase in a magnesium ion buffer was too inefficient to be practical, PCR amplification starting with an RNA template initially was carried out by first reverse-transcribing the target RNA using, for example, a non-thermostable viral reverse transcriptase such as Molony Murine Leukemia Virus Reverse Transcriptase (MoMuLV RT) or AMV-RT, and then amplifying the resulting cDNA using a thermostable DNA polymerase.

[0007] A significant advance was achieved with the discovery that a thermostable DNA polymerase could be used to efficiently reverse transcribe an RNA template by carrying out the reaction in a manganese buffer (Mn+2), rather than a magnesium (Mg+2) buffer, as is preferred for primer extension using a DNA template. Efficient Mn+2-activated reverse transcription using a thermostable DNA polymerase is described in U.S. Patent Nos. 5,310,652; 5,322,770; 5,407,800; 5,641,864; 5,561,058; and 5,693,517, all incorporated herein by reference. As both the synthesis of cDNA from an RNA template and the synthesis of DNA from a DNA template can be carried out in a Mn+2 buffer, the use of a Mn+2 buffer enables single-enzyme, coupled reverse transcription/amplification reactions (see also Myers and Sigua, 1995, in PCR Strategies, supra, chapter 5).

SUMMARY OF INVENTION

[0008] The present invention provides methods of reverse transcribing RNA sequences using a thermostable DNA polymerase. The present invention further provides methods for reverse transcribing and amplifying RNA sequences, preferably using a single thermostable DNA polymerase in a coupled, one-tube reaction. The methods of the present invention provide improved reverse transcription ("RT") efficiency relative to previously described high-temperature

reverse transcription methods.

15

25

30

35

40

45

50

[0009] In a preferred embodiment, the invention provides methods of reverse transcribing RNA sequences in a magnesium ion (Mg+2) buffer and, further, methods for reverse transcribing and amplifying RNA sequences using a single thermostable DNA polymerase in a Mg+2 buffer, preferably in a coupled, one-tube reaction. The methods carried out using a Mg+2 buffer provide enhanced fidelity over previously described methods that rely on manganese (Mn+2) activation of a thermostable DNA polymerase.

[0010] The methods of the present invention use a mutant thermoactive, preferably thermostable, DNA polymerase that contains a point mutation in a critical amino acid position previously described as affecting the DNA polymerase's ability to incorporate dideoxynucleotides (ddNTP's) labeled with fluorescein or cyanine family dyes. The present invention results from the surprising discovery that these mutant DNA polymerases also exhibit a significantly increased ability to carry out reverse transcription, particularly in a Mg+2 buffer.

[0011] Mutant DNA polymerases useful in the methods of the present invention are described in European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496, each incorporated herein by reference. These mutant DNA polymerases are described as exhibiting an increased ability to incorporate nucleotides, including deoxynucleotides (dNTP's) and base analogues such as dideoxynucleotides (ddNTP's), labeled with fluorescein and cyanine family dyes. For convenience, these mutant DNA polymerases are referred to herein as "fluorescein family dye incorporating" DNA polymerases, or "FDI" DNA polymerases. The primary utility described for FDI DNA polymerases is in DNA sequencing reactions that use dye-terminators (dye labeled ddNTP's) labeled with fluorescein or cyanine family dyes. Because a wild-type DNA polymerase discriminates against nucleotide analogues, and even more so against labeled nucleotide analogues, dye-terminator sequencing reactions typically were carried out using an excess of dye-terminators. By decreasing the discrimination against the labeled dye-terminators, FDI DNA polymerases permit sequencing reactions to be carried out with a significantly lower concentration of dye-terminators.

[0012] The critical amino acid position that is mutated in the DNA polymerases used in the present methods, which is the same critical amino acid that affects the DNA polymerase's ability to incorporate dideoxynucleotides labeled with fluorescein and cyanine family dyes, is identified in European Patent Publication No. 0 902,035, and co-pending U.S. Application No. 09/146,631 by its location within a conserved sequence motif present in the native form of the enzyme. Examples of the sequence motif in a number of DNA polymerases is provided therein in Table 1. The sequence motif and the critical amino acid are identified below in essentially the same manner.

[0013] Described most generally, using the standard single-letter abbreviations for amino acids, this critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LXXXXXXXXE (SEQ ID NO: 1),

wherein X at position 2 is S or A, X at positions 3, 4, 6, 7, 8, 9, and 10 are any amino acid, and X at position 5 is L or I. [0014] In a more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSXELXIPYEE (SEQ ID NO: 2),

wherein X at position 3 is Q or G, and X at position 6 is S or A. Examples of DNA polymerases containing this motif are DNA polymerases from the genus *Thermus*.

[0015] In a preferred embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSQELAIPYEE (SEQ ID NO: 3).

Examples of DNA polymerases containing this motif are DNA polymerases from Thermus species aquaticus, thermophilus, ZO5, and caldophilus.

[0016] In another preferred embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSXELSIPYEE (SEQ ID NO: 4),

wherein X at position 3 is Q or G. Examples of DNA polymerases containing this motif are DNA polymerases from Thermus species flavus, sps17, and filiformis.

[0017] In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

10

15

LSVRLGXPVKE (SEQ ID NO: 5);

wherein X at position 7 is V or I. Examples of DNA polymerases containing this motif are DNA polymerases from *Thermotoga* species *maritima* and *neopolitana*.

[0018] In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

20

LSKRIGLSVSE (SEQ ID NO: 6).

An example of a DNA polymerase containing this motif is the DNA polymerases from *Thermosipho africanus*. **[0019]** In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LAQNLNIXRKE (SEQ ID NO: 7),

30

35

40

wherein X at position 8 is S or T. Examples of DNA polymerases containing this motif are DNA polymerases from *Bacillus* species *caldotenax* and *stearothermophilus*.

[0020] In each of the critical motifs identified above, the critical amino acid is at amino acid position 4.

[0021] As demonstrated in the examples, mutation of the critical amino acid to any amino acid other than E (present in the native DNA polymerase used in the examples), A, G, or P provided improved RT efficiency. Thus, the methods of the present invention use a thermoactive, preferably thermostable, mutant DNA polymerase characterized in that the native form of the DNA polymerase comprises a sequence motif selected from the group consisting of SEQ ID NOs: 1 - 7, and the amino acid at position 4 of the motif is mutated to any amino acid other than E, A, G, or P. Preferably, the critical amino acid is mutated to any amino acid other than E, A, G, P, Q, or D.

[0022] One aspect of the invention relates to methods of reverse transcribing an RNA, which comprises carrying out a reverse transcription reaction using a mutant thermoactive or thermostable DNA polymerase as described herein. Preferably, the methods comprise:

- (a) providing a reaction mixture comprising a primer sufficiently complementary to the RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to the target RNA and a mutant thermoactive or thermostable DNA polymerase as described herein; and
 - (b) treating the reaction mixture under appropriate conditions to provide single-stranded cDNA.

50

55

[0023] In a preferred embodiment, the reaction of step (a) is carried out in an appropriate buffer, wherein the buffer comprises Mg+2.

[0024] Another aspect of the invention relates to methods for amplifying an RNA, which comprise carrying out a single-enzyme, combined reverse transcription/amplification reaction using a mutant thermoactive or thermostable DNA polymerase as described herein. Preferably, the methods comprise:

(a) providing a reaction mixture comprising a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to

the target RNA, and the second primer is sufficiently homologous to the target RNA to hybridize to the cDNA and initiate synthesis of an extension product, and a mutant thermoactive or thermostable DNA polymerase as described herein, in an appropriate buffer, wherein the buffer comprises Mg+2;

- (b) treating the reaction mixture under appropriate conditions to provide single-stranded cDNA; and
- (c) treating the reaction mixture of step (b) under appropriate conditions to amplify the cDNA of step (b).

[0025] In a preferred embodiment, the reverse transcription and amplification is carried out as a single-enzyme, onetube, coupled reverse transcription/PCR amplification reaction using a mutant thermoactive or thermostable DNA polymerase as described herein in a buffer comprising Mg+2.

DETAILED DESCRIPTION OF THE INVENTION

5

20

[0026] To aid in understanding the invention, several terms are defined below.

[0027] The term "thermoactive DNA polymerase", as used herein, refers to a DNA polymerase that has an elevated temperature reaction optimum. The thermoactive enzyme used in the present invention catalyzes primer extension optimally at a temperature between 60 and 90°C.

[0028] The term "thermostable DNA polymerase" refers to a DNA polymerase that is stable to heat, i.e., does not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. The heating conditions necessary for nucleic acid denaturation are well known in the art.

As used herein, a thermostable polymerase is suitable for use in a temperature cycling reaction such as the polymerase chain reaction ("PCR") amplification methods described in 4,965,188, incorporated herein by reference.

[0029] A "high-temperature reverse transcription reaction", as used herein, refers to a reverse transcription reaction carried out at a temperature at least 40°C, preferably, 40°C-80°C, and more preferably 50°C-70°C.

[0030] The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a recoverable bioactive polypeptide or precursor.

[0031] The term "native" refers to a gene or gene product which is isolated from a naturally occurring source. This term also refers to a recombinant form of the native protein produced by molecular biological techniques which has an amino acid sequence identical to that of the native form.

[0032] The term "mutant" refers to a gene that has been altered in its nucleic acid sequence or a gene product which has been altered in its amino acid sequence, resulting in a gene product which may have altered functional properties when compared to the native or wild-type gene or gene product. Such alterations include point mutations, deletions and insertions

[0033] As used herein, a "point mutation" in an amino acid sequence refers to either a single amino acid substitution or single amino acid deletion. A point mutation preferably is introduced into an amino acid sequence by a suitable codon change in the encoding DNA.

[0034] Individual amino acids in a sequence are represented herein as AN, wherein A is the amino acid in the sequence and N is the position in the sequence. Substitution-type point mutations within an amino acid sequence are represented herein as A₁NA₂, wherein A₁ is the amino acid in the unmutated protein sequence, A₂ is the amino acid in the mutated protein sequence, and N is the position in the amino acid sequence. Either the one-letter or three-letter codes are used for designating amino acids (see Lehninger, Biochemistry 2nd ed., 1975, Worth Publishers, Inc. New York, NY: pages 73-75, incorporated herein by reference). For example, a G46D mutation represents a change from glycine to aspartic acid at amino acid position 46. The amino acid positions are numbered based on the full-length sequence of the protein from which the region encompassing the mutation is derived. Representations of nucleotides and point mutations in DNA sequences are analogous.

[0035] The terms "nucleic acid" and "oligonucleotide" refer to primers, probes, and oligomer fragments to be detected, and shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. There is no intended distinction in length between the terms "nucleic acid" and "oligonucleotide", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA.

[0036] Oligonucleotides can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang et al., 1979, Meth. Enzymol. 68:90-99; the phosphodiester method of Brown et al., 1979, Meth. Enzymol. 68:109-151; the diethylphosphoramidite method of Beaucage et al., 1981, Tetrahedron Lett. 22:1859-1862; and the solid support method of U.S. Patent No. 4,458,066, each incorporated herein by reference. Automated synthesis using cyanoethyl

phosphoramidite chemistry is preferred. Reagents and instruments are commercially available from, for example, PE Biosystems (Applied Biosystems Foster City, CA) and Pharmacia (Piscataway, NJ).

[0037] The term "primer", as used herein, refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. A primer is preferably a single-stranded oligodeoxyribonucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 35 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template for primer elongation to occur.

[0038] A "pair of primers", as used herein, refers to a first and second primer selected to function in an amplification reaction, such as a polymerase chain reaction, to amplify a desired target sequence. For example, for use in a coupled reverse transcription/amplification reaction to amplify a target RNA, a pair of primers comprises a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to the target RNA, and said second primer is sufficiently homologous to said target RNA to hybridize to the cDNA and initiate synthesis of an extension product. The design of primer pairs for the amplification of nucleic acid sequences is well known in the art.

10

15

20

30

35

40

[0039] A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ³²P, fluorescent dyes, electrondense reagents, enzymes (as commonly used in ELISA assays), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available.

[0040] As used herein the term "cDNA" refers to a copy DNA molecule synthesized using a ribonucleic acid strand (RNA) as a template. The RNA may be mRNA, tRNA, rRNA, or another form of RNA, such as viral RNA. The cDNA may be single-stranded, double-stranded or may be hydrogen-bonded to a complementary RNA molecule as in an RNA/cDNA hybrid.

[0041] The term "reverse transcription reaction mixture" refers to an aqueous solution comprising the various reagents used to reverse transcribe a target RNA. These include enzymes, aqueous buffers, salts, oligonucleotide primers, target nucleic acid, and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete reverse transcription reaction mixture.

[0042] The term "amplification reaction mixture" refers to an aqueous solution comprising the various reagents used to amplify a target nucleic acid. These include enzymes, aqueous buffers, salts, amplification primers, target nucleic acid, and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete amplification reaction mixture. In the preferred embodiment of the invention, the amplification reaction is a polymerase chain reaction (PCR) and the amplification reaction mixture is a PCR mixture. As used herein, an amplification reaction mixture encompasses the reaction mixture used for the amplification of an RNA, as in a coupled reverse transcription/amplification reaction.

[0043] The term "buffer," as used herein, refers to a solution containing a buffering agent or a mixture of buffering agents and, optionally, a divalent cation and a monovalent cation.

[0044] Conventional techniques of molecular biology and nucleic acid chemistry, which are within the skill of the art, are fully explained in the literature. See, for example, Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Nucleic Acid Hybridization (B.D. Hames and S.J. Higgins. eds., 1984); Basic Methods in Molecular Biology (Elsevier, NY); Current Protocols in Molecular Biology (John Wiley and Sons, NY); and a series, Methods in Enzymology (Academic Press, Inc.), all of which are incorporated herein by reference. All patents, patent applications, and publications mentioned herein, both supra and infra, are incorporated herein by reference.

[0045] The mutant DNA polymerases used in the methods of the present invention contain a point mutation at a critical amino acid position identified in European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496, each incorporated herein by reference. European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631 identify the critical amino acid in terms of its position within a conserved critical sequence motif found in the native DNA polymerase sequence. PCT International Patent Publication No. WO 98/40496 identifies the critical amino acid in Taq DNA polymerase by position number (E681) and in other DNA polymerase by amino acid sequence homology to Taq DNA polymerase. Both methods identify the same critical amino acid position. For clarity of description, the critical amino acid is described herein in terms of its position in the conserved critical sequence motif.

[0046] Table 1, reproduced in essence from Table 1 of European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631, provides the critical motifs found in a number of representative DNA polymerases (the critical amino acid is highlighted) along with positions of the critical amino acid within the full enzyme sequence. Multiple position numbers are provided where slightly different amino acid sequences for DNA polymerases from the same species have been reported in the literature.

TABLE 1

Organism	SEQ ID NO:	Critical Motif	Position
Thermus			<u> </u>
aquaticus	8	LSQELAIPYEE	681
flavus	9	LSGELSIPYEE	679
thermophilus	10	LSQELAIPYEE	683
species Z05	11	LSQELAIPYEE	683
species sps17	12	LSQELSIPYEE	679
caldophilus	13	LSQELAIPYEE	683
filiformis	14	LSQELSIPYEE	679
Thermotoga			<u> </u>
maritima	15	LSVRLGVPVKE	744
neapolitana	16	LSVRLGIPVKE	744
Thermosipho			
africanus	17	LSKRIGLSVSE	743
Bacillus			
caldotenax	18	LAQNLNISRKE	725, 724
stearothermophilus	19	LAQNLNITRKE	724, 727, 80

[0047] A number of species possessing a thermoactive DNA polymerase having the critical motif are described in European Patent Publication No. 0 902 035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496. Preferred DNA polymerases for use in the present invention are derived from a *Thermus* species.

[0048] The mutant DNA polymerase used in the methods of the present invention is a thermoactive, preferably thermostable, mutant DNA polymerase characterized in that the native form of the DNA polymerase comprises a sequence motif selected from the group consisting of SEQ ID NOs: 1-7, and the amino acid at position 4 of the motif is mutated to any amino acid other than E, A, G, or P. Preferably, the critical amino acid is mutated to any amino acid other than E, A, G, P, or Q, more preferably to any amino acid other than E, A, G, P, Q, or D.

[0049] The mutant DNA polymerase can be derived from any species possessing a thermoactive DNA polymerase having the critical motif in the polymerase domain. The critical motif identifies a particular functional region within the polymerase domain of the enzyme, and identifies an amino acid within the motif that is critical to the function. The examples describe the effects on Mg+2-activated reverse transcription efficiency of each possible mutation at this site in one widely used thermostable DNA polymerase, *Thermus thermophilus* DNA polymerase. Just as amino acid changes at this site affect the efficiency of incorporation of dideoxynucleotides (ddNTP's) labeled with fluorescein or cyanine family dyes in essentially all DNA polymerases having the conserved critical motif, it is expected that amino acid changes at this site will affect the Mg+2-activated reverse transcription efficiency of essentially all DNA polymerases having the conserved critical motif.

[0050] The structural relatedness of DNA polymerases and the presence of conserved functional domains is well known (see, for example, Ito and Braithwaite, 1991, Nucl. Acids Res. 19(15):4045-4-47; Blanco et al. 1991, Gene 100: 27-38; Gutman and Minton, 1993, Nucl. Acids. Res. 21(18):4406-4407; and Delarue et al., 1990, Protein Engineering 3(6):461-467; each incorporated herein by reference). Mutations of a critical amino acid within a conserved functional domain, in general, are expected to have analogous effects when introduced into other DNA polymerases (see, for example, Xu et al., 1997, J. Mol. Biol. 268:284-302; U.S. Patent Nos. 5,466,591; 5,795,762; 5,939,292; and 5,614,365; each incorporated herein by reference).

[0051] Additional thermoactive or thermostable DNA polymerases containing the critical motif, and the position of the critical amino acid therein, can be identified routinely by direct inspection of the amino acid sequence. Additionally, the critical motif and amino acid can be identified by sequence homology with another DNA polymerase known to contain the critical motif, such as the DNA polymerases from the *Thermus* species listed in Table 1. Amino acid and nucleic acid sequence alignment programs are readily available. For example, widely used sequence alignment programs, including "GAP," "BESTFIT," and "PILEUP," are available from the Genetics Computer Group (Madison, Wisconsin). In general, carrying out a sequence alignment using the default parameters facilitates identification of the critical amino acid in a DNA polymerase sequence homologous to the critical amino in one of the DNA polymerases

listed in Table 1.

10

15

20

30

35

40

45

50

[0052] As new DNA polymerase sequences are obtained, sequences may be discovered that contain a variant of the critical motif that is not literally described by SEQ ID NO: 1, but is identifiable by sequence homology with the known enzymes. As a hypothetical example, an enzyme having a motif in the DNA polymerase domain that is a variant of SEQ ID NO: 3, differing only in that the amino acid at position 5 is other than L or I, would be recognized as having the critical motif in view of the high homology (10 out of 11 amino acids in this example) with the critical motif of several *Thermus* species enzymes. Such an enzyme is considered equivalent for the purposes of the present invention.

[0053] The critical amino acid is identified with reference to the native enzyme. However, this is not meant to restrict the amino acid sequence of the mutant enzyme everywhere else to that of the native enzyme. Mutant DNA polymerases used in the methods of the present invention may contain additional mutations whose presence may be advantageous for particular applications. For example, mutations which eliminate 5' to 3' exonuclease activity or 3' to 5' exonuclease activity and their applications are well known. An additional substitution mutation in position 3 of the critical motifs identified as SEQ ID NOs: 1-7 (for example, a Q682K, E683K mutant of Tth DNA polymerase) may provide additional benefits, particularly in Mn+2-activated reactions, such as allowing a further reduction of the Mn+2 concentration or further broadening the range of usable salt concentrations.

[0054] Mutant DNA polymerases for use in the methods of present inventions preferably are expressed from recombinant expression vectors in which the coding sequence has been modified to express the particular mutant protein sequence of interest. Methods for introducing point mutations into a coding sequence in an expression plasmid are well known in the art and described in the patent and scientific literature incorporated herein by reference. Detailed protocols are provided in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989, second edition, chapter 15.51, "Oligonucleotide-mediated mutagenesis," and Ausebel et al., Current Protocols in Molecular Biology (current edition), both of which are incorporated herein by reference. European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496 teach the construction of appropriate expression vectors and the expression and purification of the resulting mutant DNA polymerase. Following the guidance provided in the cited references, and using only well known techniques, one skilled in the art will be able to prepare any number of expression vectors containing a mutant gene suitable for expressing in any of a variety of host systems mutant DNA polymerases for use in the methods of the present invention.

[0055] For use in the present high temperature reverse transcription methods, it is only essential that the DNA polymerase is thermoactive. Because the preparation of cDNA from an RNA template does not involve repeated denaturation cycles at elevated temperatures, it is not essential that enzymes useful in the method are thermostable as well as thermoactive. In the single enzyme, combined reverse transcription/polymerase chain reaction amplification (RT/PCR) methods described in the examples, use of a thermostable DNA polymerase is preferred because the DNA polymerase is subject both to RT conditions and to the PCR conditions, which included repeated denaturation cycles. [0056] For reverse transcription, according to the present invention, the reaction is carried out in a mixture containing the RNA template, a primer, and a thermoactive or thermostable mutant DNA polymerase. The reaction mixture typically contains all four deoxyribonucleotide triphosphates (dNTPs) and a buffer containing a divalent cation and a monovalent cation. DNA polymerases require a divalent cation for catalytic activity. For extension reactions using a DNA template, the preferred divalent cation is Mg+2, although other cations, such as Mn+2 or Co+2 can activate DNA polymerases.

[0057] In contrast to extension reactions using a thermoactive or thermostable DNA polymerase and a DNA template, extension reactions using an RNA template, i.e., reverse-transcription, essentially have required the use of Mn+2 in order to achieve useful efficiency. For example, the use of MnCl₂ or Mn(OAc)₂ for RNA amplification with Tth DNA polymerase provides an increase in sensitivity of at least 10⁶-fold compared to the use of MgCl₂ and standard PCR conditions.

[0058] The use of Mn+2, although it increases the efficiency of reverse transcription, also decreases the fidelity, resulting in an increased number of misincorporated nucleotides. The use of Mn+2 also decreases the fidelity of DNA amplifications. Thus, single-enzyme, one-tube RNA amplification reactions using Mn+2 suffer reduced fidelity in both the RNA and DNA phases of the reaction. Thus, when higher fidelity RNA amplification is desired, it is preferable to carry out the reaction in two stages. This is achieved by effectively removing the Mn+2 ions from the reverse-transcription mixture using a chelator, such as EDTA or, preferably, EGTA, and then adding an appropriate Mg+2-containing DNA amplification mixture to complete the reaction.

[0059] The use of the mutant DNA polymerases in the methods of the present invention provide benefits for reverse transcription reactions regardless of the divalent cation used. In Mn+2 reactions, the use of the mutant DNA polymerase provides for high temperature reverse-transcription and amplification of RNA with a higher efficiency than achieved using the native enzyme. In addition, the use of the mutant DNA polymerase allows carrying out the reaction at a lower Mn+2 concentration, thereby minimizing the deleterious effect of Mn+2 concentration on fidelity.

[0060] Particularly surprising is that the mutant DNA polymerases enable reverse transcription to be carried out using Mg+2 with significantly increased efficiency. The use of Mg+2, the enzyme's preferred divalent cation, provides for

significantly higher fidelity. Thus, in Mg⁺² reactions, the use of the mutant DNA polymerase provides for high temperature, high fidelity reverse-transcription and amplification of RNA with a usable efficiency.

[0061] The divalent cation is supplied in the form of a salt such MgCl₂, Mg(OAc)₂, MgSO₄, MnCl₂, Mn(OAc)₂, or MnSO₄. In general, for reactions using Mn+², usable cation concentrations in a Tris-HCl buffer will be in a range from 0.5 to 7 mM MnCl₂, preferably between 0.5 and 2 mM, and in a bicine/KOAc buffer or tricine/KOAc buffer will be in a range from 0.5 to 20 mM Mn(OAc)₂, preferably between 0.5 and 5 mM. In general, for reactions using Mg+², usable divalent cation concentrations in a Tris-HCl buffer will be in a range from 0.5 to 10 mM MgCl₂, and in a bicine/KOAc or tricine/KOAC buffers, will be in a range from 0.5 to 20 mM for Mg(OAc)₂, preferably between 0.5 and 5 mM. These concentrations provide useful starting conditions for carrying out routine reaction optimization. The optimal divalent ion concentration in a particular reaction will depend not only on the particular enzyme used, but also on the other reaction components, such as, for example, the dNTP concentration and primer sequence and concentration. One of skill will understand that reaction conditions in general, and the divalent cation concentration in particular, can be optimized empirically for any particular reaction using routine experimental methods.

[0062] Previously, while capable of activating RNA template-directed DNA synthesis, mixed divalent cation buffers (e.g., Mn+2 and Mg+2), were not preferred due to reduced sensitivity and efficiency. It is expected that mixed divalent cation buffers are useful in the methods of the present invention and, in some applications, may be preferable. Mixed cation use may enable, for example, a tradeoff between a higher efficiency, but lower fidelity Mn+2-activated reaction and a higher fidelity Mg+2-activated reaction.

[0063] High temperature reverse transcription methods and combined reverse transcription/amplification methods using a thermostable DNA polymerase in a Mn⁺² buffer are well known in the art. See, for example, U.S. Patent Nos. 5,310,652; 5,322,770; 5,407,800; 5,561,058; 5,641,864; and 5,693,517; each incorporated herein by reference. The methods of the present invention represent a modification of the previously described methods, wherein the modification involves the use of mutant DNA polymerases, as described above. In a preferred embodiment, the reaction is carried out in buffer containing Mg⁺² as the divalent cation used to activate the DNA polymerase.

[0064] One advantage of the present methods is that the use of the mutant DNA polymerases appears to provide faster RT extension rates and, consequently, less time is needed for the RT reaction. Preferably, to maximize the amount of cDNA produced in a reverse transcription reaction, the reaction is carried out for about 30 minutes. Depending on the application, particularly in manganese reactions, RT times as short as one minute or less may provide acceptable results.

[0065] Other advantages of the present methods are that the use of the mutant DNA polymerases may provide improved RT efficiency at lower enzyme concentrations and, furthermore, provide a wider range of usable salt concentrations. It is expected that optimal reaction conditions will depend on, for example, the particular enzyme used and can be determined empirically in a routine manner.

[0066] Other aspects required to carry out the present methods, such as selection of a target RNA, sample preparation, primer design, and choice of reagents and reaction conditions other than the DNA polymerase and divalent cation used to activate the DNA polymerase are well known in the art and described in, for example, the above-referenced patents. Similarly, if the reverse transcription is coupled with an amplification reaction, all aspects of the amplification not relating to the DNA polymerase and divalent cation used to activate the DNA polymerase are well known in the art and described in, for example, the above-referenced patents. Finally, applications of reverse transcription and amplification of RNA are well known in the art and described in, for example, the above-referenced patents. One of skill in the art will be able to apply the present methods in any application in which the reverse transcription and, optionally, amplification of RNA is desired.

[0067] The following examples are offered by way of illustration only and should not be construed as intending to limit the invention in any manner.

EXAMPLE 1

20

25

30

35

40

45

EXAMPLES OF MUTANT DNA POLYMERASE

[0068] A series of 19 mutant DNA polymerases were constructed from "native" *Thermus thermophilus* (Tth) DNA polymerase representing all possible mutations in the critical amino acid. As described in European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631, Tth DNA polymerase amino acid sequence contains the critical sequence motif represented as SEQ ID NO: 3 (which is a particular embodiment of SEQ ID NO: 2, which is a narrower embodiment of the general motif, SEQ ID NO: 1). The critical amino acid is at position 683 (E683).

[0069] The sequence of Tth DNA polymerase and plasmids containing the gene for Tth DNA polymerase are known in the art (see, for example, U.S. Patent Nos. 5,618,711 and 5,789,224, both incorporated herein by reference). The particular plasmid used in the present example encodes a Tth DNA polymerase also containing a G46E point mutation that eliminates the 5' to 3' exonuclease activity of the enzyme, as described in U.S. Patent No. 5,466,591, incorporated

herein by reference. In addition, the plasmid contains silent nucleotide substitutions that introduce a *Cla*I recognition and cleavage site encompassing codons 678, 679, and the first nucleotide of 680, without changing the encoded amino acid sequence. The presence of the additional mutation in the 5' to 3' exonuclease domain is believed to have no appreciable effect on the ability of the DNA polymerase to reverse transcribe RNA in a Mg+2 buffer; Tth DNA polymerase having the G46E mutation is considered herein as the native DNA polymerase.

[0070] Point mutations in the expressed proteins were introduced by mutating the encoding DNA sequence using standard techniques. Essentially, a short fragment of the coding sequence encompassing codon 683 was replaced with a synthetic fragment containing the desired sequence. The short fragment, ~65 nucleotides in length, was excised by digesting the plasmid with restriction enzymes Clal and HindIII. A synthetic double-stranded DNA insert was synthesized encoding the same amino acid sequence as the excised fragment, but containing the desired mutation in codon 683. The synthetic fragment was then ligated into the digested plasmid, yielding a plasmid containing a mutated codon encoding a full length Tth DNA polymerase having the desired point mutation.

EXAMPLE 2

15

20

25

30

35

40

50

55

REVERSE TRANSCRIPTION/AMPLIFICATION EFFICIENCY

[0071] The 20 DNA polymerases described in Example 1 (1 native and 19 mutants) were compared for their ability to catalyze reverse transcription/amplification reactions. In overview, coupled, single-enzyme reverse transcription/amplification reactions were carried out with each of the DNA polymerases. The same initial target copy number was used for each reaction, and the synthesis of amplification product was monitored during the reaction. The number of cycles required to generate an arbitrary, but fixed, quantity of amplified product, which provides a measure of the reaction efficiency, was determined for each DNA polymerase. Because the initial reverse transcription step typically is the critical limiting step in a reverse transcription/amplification reaction, an improvement in overall reaction efficiency also suggests an improvement in the initial reverse transcription step.

[0072] The increase in amplified nucleic acid during the reaction was monitored using the methods described in Higuchi et al., 1992, Bio/Technology 10:413-417; Higuchi et al., 1993, Bio/Technology 11:1026-1030; Higuchi and Watson, in PCR Applications, supra, Chapter 16; U.S. Patent No. 5,994,056; and European Patent Publication Nos. 487,218 and 512,334, each incorporated herein by reference. These methods, referred to herein as kinetic-PCR, rely on the increased fluorescence that ethidium bromide (EtBr) and other DNA-binding dyes exhibit when bound to double-stranded DNA in order to detect the change in amount of double-stranded DNA in a reaction. The increase of double-stranded DNA resulting from the synthesis of target sequences results in an increase in the amount of dye bound to double-stranded DNA and a concomitant detectable increase in fluorescence.

[0073] Amplifications were carried out with ethidium bromide in the reaction. Alternatively, amplifications can be carried out using SYBR® Green I (Molecular Probes, Eugene, OR) in the reaction. Both dyes increase their fluorescence upon intercalation into, or binding to, the double-stranded DNA. The reactions are carried out in a combined thermal cycler and fluorescence detection system which enables monitoring the fluorescence of the reaction mixture during the amplification. It will be clear that, in addition to the instruments described below, any suitable instrument can be used.

RNA TARGET AND AMPLIFICATION PRIMERS

[0074] A target RNA was synthsized using an expression plasmid encoding the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. A region of the GAPDH RNA was amplified using the following primers, shown in the 5' to 3' orientation:

P1 (SEQ ID NO: 20) 5'-CGAGATCCCTCCAAAATCAA

P2 (SEQ ID NO: 21) 5'-CATGAGTCCTTCCACGATACCAA

[0075] The initial target concentration was measured by standard means. For the comparison reactions described herein, the absolute copy number is less important than the relative copy number. To insure the same initial copy number in each reaction, aliquots of dilutions of the same initial RNA stock were used.

[0076] One of skill will recognize the selection of target is a matter of convenience. Other RNA targets and corresponding amplification primers could be used in essentially the same protocol. Routine optimization of the reaction conditions would be expected.

5 AMPLIFICATION

10

15

20

25

35

45

50

55

[0077] Each RT-PCR amplification was carried out in a total reaction volume of 100 µl. The final reagent concentrations were as follows:

10 units DNA polymerase
10⁶ copies GAPDH RNA
50 mM Tricine, pH 8.15
50 mM KOAc
2 mM Mg(OAc)₂
200 μM dATP, dGTP, dCTP
400 μM dUTP
200 nM each primer
8% glycerol
1% DMSO
1 μg/ml of ethidium bromide
2 units UNG*

[0078] Alternatively, SYBR® Green I can be used instead of ethidium bromide to enable detection of the amplified product. Amplifications using SYBR® Green I are carried out with 0.2 X SYBR® Green I (sold as 10,000 X) diluted in DMSO.

[0079] Reactions using ethidium bromide preferably are carried out using a ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), which allows the selection of suitable detection wavelengths. Reactions using SYBR® Green I preferably are carried out using a GeneAmp® 5700 Sequence Detection Systems, Foster City, CA) using the same thermal cycling conditions. The GeneAmp® 5700 Sequence Detection System is designed for use with SYBR® Green I and the excitation and detection wavelengths are pre-set for this dye.

[0080] The assays described below were carried out using a custom instrument consisting essentially of a GeneAmp® PCR system 9600 thermal cycler (PE Biosystems, Foster City, CA) modified by the addition of a fluoresence detection system similar to that used in the GeneAmp® 5700 Sequence Detection System, but designed for use with ethidium bromide. Results obtained using the custom instrument would be expected to be comparable to results obtained using one of the preferred instruments.

[0081] Amplification reactions were carried out using the specific temperature cycling profile shown below.

40 THERMAL CYCLING TIMES AND TEMPERATURES

[0082]

Pre-reaction	incubation:	50°C for 2 minutes
Reverse-transcription		60°C for 30 minutes
		95°C for 1 minute
55 cycles	denature:	95°C for 15 seconds
	anneal:	55°C for 30 seconds
	extend:	72°C for 15 seconds
Final extens	ion and hold:	72°C

DETECTION

[0083] The accumulation of amplified product was measured at each cycle during the reaction by measuring the increase in reaction fluorescence. During each amplification cycle, each reaction was excited with light at a wavelength

^{*} Manufactured by Roche Molecular Systems and commercially available through Applied Biosystems, Foster City, CA.

near the excitation maximum of the dye and the emission of the dye was measured near its emission maximum.

[0084] Fluorescence measurements were normalized by dividing by an initial fluorescence measurement obtained during a cycle early in the reaction while the fluorescence measurements between cycles appear to be relatively constant. The cycle number chosen for the initial fluorescence measurement was the same for all reactions compared, so that all measurements represent increases relative to the same reaction cycle.

[0085] The number of amplification cycles carried out until the fluorescence exceeded an arbitrary fluorescence level (AFL) was calculated from the observed fluorescence values. The AFL was chosen close to the baseline fluorescence level, but above the range of random fluctuations in the measured fluorescence, so that the reaction kinetics were measured during the early phase of the amplification when the amount of product increases geometrically. During this geometric growth phase of the amplification, the number of cycles required to reach a particular threshold value depends solely on the initial copy number and the reaction efficiency. As each reaction is carried out using the same initial target copy number, the number of cycles to reach the threshold provides a measure of the reaction efficiency. In later cycles, accumulation of amplified product and exhaustion of reagents eventually leads to a reaction plateau. [0086] An AFL of 1.12 was chosen for all reactions. Because a PCR amplification consists of discrete cycles and the fluorescence measurements are carried out once per cycle, the measured fluorescence typically increases from below the AFL to above the AFL in a single cycle. To improve the precision of the measurements, an "exact" number of cycles to reach the AFL threshold, referred to herein as the C_T value, was calculated by interpolating fluorescence measurements between cycles.

RESULTS:

20

25

30

35

40

45

50

55

[0087] The C_T values obtained using the native Tth DNA polymerase (E683) and each of the mutant DNA polymerases (identified by amino acid at position 683) are shown in the table, below. Each C_T value represents the average value obtained from two reactions. To facilitate comparison, the difference of the C_T values, (C_T native) - (C_T mutant), also is provided. An increase in efficiency using the mutant DNA polymerase results in reaching the threshold value in fewer cycles, i.e., a lower C_T value. Thus, a positive difference in C_T values reflects an increase in efficiency.

REACTION EFFICIENCIES					
aa @ 683 1-letter code aa @ 683 3-letter code			(C _T native) - (C _T mutant)		
Ε	Glu	37.7	0.0		
Α	Ala	38.7	-1.1		
С	Cys	33.2	4.5		
D	Asp	36.6	1.1		
F	Phe	28.2	9.5		
G	Gly	42.3	-4.7		
Н	His	32.0	5.7		
1	lle	33.4	4.3		
К	Lys	27.6	10.1		
L	Leu	27.8	9.9		
М	Met	30.3	7.4		
N	Asn	31.5	6.2		
Р	Pro	38.8	-1.1		
Q	Gin	37.4	0.3		
R	Arg	27.2	10.5		
S	Ser	32.0	5.7		
Т	Thr	31.5	6.2		
V	Val	31.4	6.3		
W	Trp	29.2	8.5		

(continued)

REACTION EFFICIENCIES					
aa @ 683 1-letter code aa @ 683 3-letter code Ave. C _T (C _T native) - (C _T mutant)					
Υ	Tyr	26.1	11.6		

[0088] The results indicate that mutations of the amino acid at position 683 to any amino acid except Ala, Gly, or Pro resulted in an DNA polymerase with increased efficiency. Of these, all but the Asp and Gln mutants resulted in a least a four cycle improvement in the C_T value.

EXAMPLE 3

5

10

15

25

30

35

40

45

50

55

REVERSE TRANSCRIPTION EFFICIENCY

[0089] Selected DNA polymerases described in Example 1 were compared for their ability to catalyze reverse transcription reactions. Reverse transcription reactions were carried out with each of the DNA polymerases using either Mg+2 or Mn+2. The resulting cDNA from each of the reactions then was amplified under identical conditions using the native enzyme and Mg+2. This protocol allows measuring the effect of the enzyme specifically on the reverse transcription portion of a reverse transcription/amplification reaction.

[0090] In addition to the native enzyme, reactions were carried out using DNA polymerases having mutations to F, K, L, R, and Y at amino acid position 683. Each of these mutations was shown in Example 2 to provide significant increases in efficiency in a combined reverse transcription/amplification reaction.

REVERSE TRANSCRIPTION

[0091] Each reverse transcription was carried out in a total reaction volume of 100 μl. The final reagent concentrations were as follows:

5 units DNA polymerase
10⁶ copies GAPDH RNA
50 mM Tricine, pH 8.15
50 mM KOAc
2 mM Mg(OAc)₂ or Mn(OAc)₂
200 µM dATP, dGTP, dCTP, dUTP
200 nM each primer
8% glycerol
1% DMSO
0.2 X SYBR® Green I
1 unit UNG*

[0092] The reverse trancription reactions were carried out using the specific temperature cycling profile used is shown

REVERSE TRANSCRIPTION TIMES AND TEMPERATURES

[0093]

Pre-reaction incubation: 50°C for 2 minutes
Reverse-transcription 60°C for 30 minutes
Hold: 4°C

AMPLIFICATION

[0094] Following reverse transcription, 10 μ l of the reaction products were added to 10 μ l 2 mM EGTA to chelate the

^{*} Manufactured by Roche Molecular Systems and commercially available through Applied Biosystems, Foster City, CA.

metal cation, thereby effectively removing it from the following amplification reaction. The mixture was added to a PCR amplification mixture containing the native enzyme and Mg+2. Thus, residual mutant DNA polymerase was diluted such that any effects were expected to be negligible. The PCR amplifications were carried out in 100 µl reactions with the following final reagent concentrations:

> 5 units native DNA polymerase 50 mM Tricine, pH 8.15 50 mM KOAc 2 mM Mg(OAc)₂ 200 µM dATP, dGTP, dCTP, dUTP 200 nM each primer 8% glycerol 1% DMSO 0.2 X SYBR® Green I

[0095] Amplification reactions were carried out using the specific temperature cycling profile shown below.

AMPLIFICATION THERMAL CYCLING TIMES AND TEMPERATURES

[0096]

5

10

15

20

30

40

45

95°C for 1 minute 55 cycles denature 95°C for 15 seconds anneal 55°C for 30 seconds extend 72°C for 15 seconds Final extension and hold

RESULTS

[0097] The C_T values obtained using the native Tth DNA polymerase (E683) and each of the mutant DNA polymerases (identified by amino acid at position 683) for the reverse transcription, and the native Tth DNA polymerase for all amplifications are shown in the table, below. Each C_T value represents the average value obtained from two reactions. To facilitate comparison, the difference of the C_T values, $(C_T$ native) - $(C_T$ mutant), also is provided. An increase in efficiency using the mutant DNA polymerase results in reaching the threshold value in fewer cycles, i.e., a lower C_T value. Thus, a positive difference in C_T values reflects an increase in efficiency.

REACTION EFFICIENCIES					
	MG+2-AC	CTIVATED RT			
aa @ 683 Ave. C _T (C _T native) - (C _T mutant)					
E	33.8	0.0			
F	26.9	6.9			
K	29.0	4.8			
L	26.0	7.8			
R	25.9	7.9			
Y	24.5	9.3			

55

50

REACTION EFFICIENCIES						
	MN+2-AC	CTIVATED RT				
aa @ 683	aa @ 683 Ave. C _T (C _T native) - (C _T mutant)					
Ė	24.6	0.0				
F	21.1	3.5				
K	20.2	4.4				
L	20.6	4.0				
R	20.7	3.9				
Υ	20.3	4.3				

[0098] Each of these mutant DNA polymerase provided a significantly increased efficiency in reverse transcription/amplification reactions. Because the DNA amplification portion of each reaction was carried out identically with the native enzyme, these results demonstrate that each of these mutant DNA polymerase provide increased efficiency in the reverse transcription portion of the reactions. The mutant DNA polymerases provided significantly improved efficiency using either cation.

[0099] The improvement was particularly pronounced using Mg^{+2} and demonstrates that the use of the mutant DNA polymerases makes Mg^{+2} -activated reactions practical. Consistent with what has been reported previously, RNA amplification using the native enzyme essentially requires the use of Mg^{+2} to achieve a usable reaction efficiency, as seen by the almost 10 cycle delay (33.8-24.6) in the C_T using Mg^{+2} . In contrast, using the E683Y mutant, for example, the efficiency of the Mg^{+2} -activated reaction was equal to that achieved using the native enzyme and Mg^{+2} .

EXAMPLE 4

5

10

15

20

30

40

FIDELITY

[0100] The fidelities of selected mutant DNA polymerases and the native enzyme were compared in several ways. The fidelities in coupled reverse-transcription/amplification reactions carried out in a Mg+2 buffer were compared to the fidelities when carried out in a Mn+2 buffer. Additionally, the fidelities of the enzymes when used in DNA amplifications were compared.

[0101] The fidelities of DNA polymerases can be compared by measuring the melting temperature (Tm) profile of amplified products generated using the enzymes. The fidelity of a DNA polymerase is reflected in the number of misincorporations occurring during strand synthesis. An amplification using a lower fidelity enzyme will result in greater heterogeneity in the resulting population of amplified sequences. To measure the heterogeneity, the amplified products are denatured, allowed to reanneal, and the Tm of the resulting duplexes is measured. Because the strands in the duplexes are combined at random from a heterogenous population of sequences, the duplexes, in general, contain a number of mismatches. The greater the sequence heterogeneity in the population of amplified products, the greater the average number of mismatches in the duplexes. These mismatches destabilize the duplexes and result in a lower measured Tm.

[0102] A melting curve for the amplified products of a kinetic-PCR reaction is carried out conveniently using the thermal cycler/fluorescence detection instrument used in the amplification. After amplification, the relationship between fluorescence and temperature is measured over a temperature range encompassing the denaturation temperature of the product. The transition between double-stranded and single-stranded molecules is reflected in a change in dye fluorescence. Thus, a melting curve can be determined conveniently. Alternatively, measurements can be carried out using standard methods, which typically involve monitoring the change in optical density, a measure of the amount of double-stranded DNA in the reaction, with a change in temperature.

[0103] The fidelity of the native DNA polymerase was compared to the fidelities of two of the mutants, DNA polymerases containing the E683K and E683N mutations, respectively. Coupled reverse transcription/amplification reactions were carried out in duplicate in both Mn+2 and Mg+2 buffers essentially as described in Example 2, but with the following changes. For the Mn+2 reactions, 2 mM Mn(OAc)₂ was used in the reaction. All reactions were carried out using 25 U of DNA polymerase.

[0104] To measure the hybridization stability profile (melting curve) of the amplified double-stranded target sequences, the fluorescence of the post-amplification reaction mixture was monitored over a range of temperatures covering at least 60°C to 80°C. As expected, the fluorescence measurements resulted in a sigmoidal melting curve. A Tm was

defined as the temperature of the inflection point in the sigmoidal melting curve, which corresponds to the temperature at which half the target is in single-stranded form.

RESULTS

5

10

15

20

25

30

35

40

45

50

55

[0105] The Tm values measured for the amplification products of the Mg+2-activated reactions and the Mn+2-activated reactions are shown in the table, below. Each measurement reported is the average of replicate reactions. All temperature are degrees Celsius.

AMPLIFICATION PRODUCT TM VALUES				
DNA Polymerase Tm, Mg+2 Tm, Mn+2				
Native	80	78		
E683K	80	76		
E683N	80	76		

[0106] Using a Mg⁺² buffer, no difference in fidelity was observed between the native and the mutant DNA polymerases. Similar reactions carried out using all 20 DNA polymerases (data not shown) confirmed that the fidelity of all the mutant DNA polymerases are identical to the fidelity of the native DNA polymerase in Mg⁺²-activated reactions.

[0107] Comparing the results obtained using a Mn+2 buffer to those obtained using a Mg+2 buffer, the fidelities of the all of the DNA polymerase was reduced, as can be seen from the lower Tm values obtained. Interestingly, the two mutant DNA polymerases exhibited an even lower fidelity than did the native enzyme when using a Mn+2 buffer, at least under these reaction conditions.

[0108] The fidelity is affected by the Mn+2 concentration. To compare the effect of Mn+2 concentration on the fidelity of the mutant and native enzymes, additional experiments were carried out using the E683K mutation using a range of Mn+2 concentrations from 0.5 to 5 mM. The results (data not shown) showed that, as expected the lowest Mn+2 concentration yielded the highest fidelity reactions using either enzyme. The fidelity of the mutant enzyme was more affected by an increased Mn+2 concentration than was the fidelity of the native enzyme. Surprisingly, however, at least in these experiments, the mutant enzyme also was most efficient at the lowest Mn+2 concentration. Thus, the use of the mutant enzyme allows carrying out the reaction at a lower Mn+2 concentration, thereby minimizing the deleterious effect of Mn+2 concentration on fidelity.

[0109] In addition, both Mn+2-activated and Mg+2-activated reactions also were carried out essentially as described above, but using DNA templates, which facilitates observing the effect of fidelity in only the DNA portion of the reaction. In all cases, the Tm value of the product of the DNA amplification was indistinguishable from the Tm value of the product of the RNA amplification.

[0110] The results, taken together with the results of the previous examples, demonstrate advantages of the methods of the present invention. Previously described high temperature reverse-transcription and amplification methods were carried out using Mn+2 to achieve adequate reaction efficiency, but suffered from a reduction in fidelity. The present invention provides several options. Using Mn+2, the use of the mutant enzyme provides for high temperature reverse-transcription and amplification of RNA with a higher efficiency than achieved using the native enzyme, and allows carrying out the reaction at a lower Mn+2 concentration, thereby minimizing the deleterious effect of Mn+2 concentration on fidelity. Using Mg+2, the use of the mutant enzyme provides for high temperature, high fidelity reverse-transcription and amplification of RNA with a usable efficiency.

SEQUENCE LISTING

5	<110>	F. Hoffmann-La Roche AG Grenzacherstrasse 124 4070 Basel SWITZERLAND
10	<120>	HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES
	<130>	5395
	<150>	US 60/198,336
15	<151>	2000-04-18
	<160>	21
	11002	••
	<170>	PatentIn version 3.0
20	<210>	1
	<211>	
		PRT
	<213>	Artificial
	<220>	
25	<223>	sequence motif
	<220>	773 T T N LYM
		VARIANT (2)(2)
		X is S or A
30	12237	1 10 U 01 A
	<220>	
		VARIANT
		(3)(3)
35	(223)	X is any amino acid
	<220>	
		VARIANT
		(4)(4)
40	<223>	X is any amino acid
	<220>	
		VARIANT
		(5)(5)
45	<223>	X is L or I
	<220>	
		VARIANT
		(6)(6)
50	12237	X is any amino acid
	<220>	
		VARIANT
		$(7) \dots (7)$
55	<223>	X is any amino acid

```
<220>
                     <221> VARIANT
                     <222> (8)..(8)
<223> X is any amino acid
 5
                     <220>
                     <221> VARIANT
10
                     <222> (9)..(9)
                     <223> X is any amino acid
                     <220>
                     <221>
                            VARIANT
15
                     <222> (10)..(10)
                     <223> X is any amino acid
                     <400> 1
20
                     Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Glu
                                                             10
                     <210> 2
                    <211> 11
<212> PRT
<213> Artificial
25
                     <220>
                    <223> sequence motif
30
                    <220>
                    <221> VARIANT
                    <222> (3)..(3)
<223> X is Q or G
                    <220>
                    <221> VARIANT
                    <222> (6)..(6)
<223> X is S or A
40
                    <400> 2
                    Leu Ser Xaa Glu Leu Xaa Ile Pro Tyr Glu Glu
                    <210> 3
<211> 11
                    <212> PRT
                    <213> Artificial
                    <220>
50
                    <223> sequence motif
                    <400> 3
                    Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
55
```

```
<210> 4
                     <211> 11
                     <212> PRT
                     <213> Artificial
 5
                     <220>
                     <223> sequence motif
                     <220>
                     <221> VARIANT
10
                     <222> (3)..(3)
                     <223> X is Q or G
                     <400> 4
15
                     Leu Ser Xaa Glu Leu Ser Ile Pro Tyr Glu Glu
                     <210> 5
                     <211> 11
<212> PRT
20
                     <213> Artificial
                     <220>
                     <223> sequence motif
25
                     <220>
                     <221> VARIANT
                    <222> (7)..(7)
<223> X is V or I
30
                    <400> 5
                     Leu Ser Val Arg Leu Gly Xaa Pro Val Lys Glu
                                                           10
                     <210> 6
35
                    <211> 11
                    <212> PRT
<213> Artificial
                    <220>
                    <223> sequence motif
40
                    <400> 6
                    Leu Ser Lys Arg Ile Gly Leu Ser Val Ser Glu 1 5 10
45
                    <210> 7
                    <211> 11
<212> PRT
                    <213> Artificial
50
                    <220>
                    <223> sequence motif
                    <220>
                    <221> VARIANT
                    <222> (8)..(8)
55
                    <223> X is S or T
```

```
<400> 7
                         Leu Ala Gln Asn Leu Asn Ile Xaa Arg Lys Glu
 5
                                          5
                         <210> 8
<211> 11
<212> PRT
10
                         <213> Thermus aquaticus
                         <400> 8
                         Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
15
                         <210> 9
                         <211> 11
<212> PRT
<213> Thermus flavus
20
                         <400> 9
                         Leu Ser Gly Glu Leu Ser Ile Pro Tyr Glu Glu
                         <210> 10
<211> 11
25
                         <212> PRT
                         <213> Thermus thermophilus
                         <400> 10
30
                         Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
                         <210> 11
                         <211> 11
<212> PRT
35
                         <213> Thermus sp. 205
                         <400> 11
                         Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
40
                                          5
                                                                10
                         <210> 12
                        <211> 11
                        <212> PRT
<213> Thermus sp. sps17
45
                         <400> 12
                        Leu Ser Gln Glu Leu Ser Ile Pro Tyr Glu Glu
50
                        <210> 13
<211> 11
                         <212> PRT
                        <213> Thermus caldophilus
                        <400> 13
```

	Leu Se	r Gln	Glu	Leu 5	Ala	Ile	Pro	Tyr	Glu 10	Glu
5	<210> <211> <212>	14 11 PRT								
	<213>	Therm	nus 1	Filis	formi	is				
10	<400>	14								
	Leu Ser 1	r Gln	Glu	Leu 5	Ser	Ile	Pro	Ту≍	Glu 10	Glu
	<210> <211>	15 11								
15	<212>	PRT								
	<213>	Therm	noto	ja ma	riti	ma				
	<400>	15								
20	Leu Sei 1	val	Arg	Leu 5	Gly	Val	Pro	Val	Lys 10	Glu
	<210>	16								
	<211> <212>	11 PRT								
25	<213>	Therm	otog	ja ne	apol	.itar	ıa			
	<400>	16								
	Leu Ser 1	. Val	Arg	Leu 5	Gly	Ile	Pro	Val	Lys 10	Glu
30	•			•						
	<210>	17								
	<211> <212>	11 PRT								
	<213>	Therm	osip	nho a	fric	anus	3			
35	<400>	17								
	Leu Ser 1	Lys	Arg	Ile 5	Gly	Leu	Ser	Val	Ser 10	Glu
40	<210>	18								
40	<211>	11								
	<212> <213>	PRT Bacil	lus	cald	loten	ax				
	<400>	18								
45	Leu Ala 1	Gln	Asn	Leu 5	Asn	Ile	Ser	Arg	Lys 10	Glu
	<210>	19								
	<211>	11								
50	<212> <213>	PRT Bacil	lus	stea	roth	ermo	phil	us		
	<400>	19								
	Leu Ala	Gln	Asn	Leu	Asn	Ile	Thr	Arg.	Lys	Glu
55	1			5					10	

	<210>	20	
	<211>	20	
5	<212>		
•		Artificial	
	<220>		
	<223>	primer	
10	<400>		
	cgagat	ccct ccaaaatcaa	20
	<210>		
15	<211>		
	<212>		
	<213>	Artificial	
	<220>		
20	<223>	primer	
20	<400>		
	catgag	teet teeaegatae caa	23
25	1		
	2		
	<u> </u>		
30	T 5395	engl Sequenz Basel.txt	
30		ongs bogdone buses. Cat	

Claims

35

45

- 40 1. A method for reverse transcribing an RNA that comprises:
 - (a) providing a reverse transcription reaction mixture comprising said RNA, a primer, a divalent cation, and a mutant thermoactive DNA polymerase, wherein said mutant DNA polymerase is **characterized in that**
 - i) in its native form said DNA polymerase comprises an amino acid sequence that is SEQ ID NO: 1;
 - ii) the amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, or P; and
- (b) treating said reaction mixture at a temperature sufficient for said mutant DNA polymerase to initiate synthesis of an extension product of said primer to provide a cDNA molecule complementary to said RNA.
 - 2. The method of claim 1, wherein in its native form said DNA polymerase comprises an amino acid sequence selected from the group of SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7.
- 55 3. The method of claim 1, wherein in its native form said DNA polymerase comprises an amino acid sequence of SEQ ID NO: 2
 - 4. The method of claim 3, wherein in its native form said DNA polymerase comprises an amino acid sequence selected

from the group of SEQ ID NO: 3 and SEQ ID NO: 4.

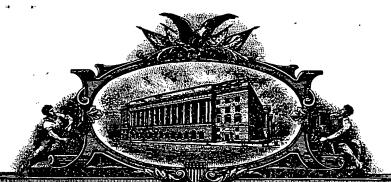
- 5. The method of any of claims 1 to 4, wherein said divalent cation is Mg+2.
- The method of any of claims 1 to 4, wherein said mutant DNA polymerase is thermostable.
 - The method of any of claims 1,3 and 4, wherein said DNA polymerase is a mutant form of a Thermus species DNA polymerase.
- The method of claim 7, wherein said DNA polymerase is a mutant form of *Thermus thermophilus* DNA polymerase or *Thermus aquaticus* DNA polymerase.
 - The method of any of claims 1 to 8, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
 - 10. The method of any of claims 1 to 9, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, P, Q, or D.
 - 11. A method for amplifying an RNA that comprises:
 - (a) reverse transcribing said RNA according to a method of claim 1 to provide a cDNA;
 - (b) amplifying said cDNA.
- 12. The method of claim 11, wherein in its native form said DNA polymerase comprises an amino acid sequence selected from the group of SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7.
 - 13. The method of claim 11, wherein in its native form said DNA polymerase comprises an amino acid sequence of SEQ ID NO: 2.
- 30 14. The method of claim 13, wherein in its native form said DNA polymerase comprises an said amino acid sequence selected from the group of SEQ ID NO: 3 and SEQ ID NO: 4.
 - 15. The method of any of claims 11 to 14, wherein in step (b) said amplifying is carried out using a polymerase chain reaction.
 - 16. The method of any of claims 11 to 15, wherein the RNA is amplified using a single-enzyme reverse transcription/amplification reaction.
 - 17. The method of any of claims 11 to 16, wherein said divalent cation is Mg+2.
 - 18. The method of any of claims 11 to 17, wherein said mutant DNA polymerase is thermostable.
 - 19. The method of any of claims 11, 13 and 14, wherein said DNA polymerase is a mutant form of a *Thermus* species DNA polymerase.
 - 20. The method of claim 19, wherein said DNA polymerase is a mutant form of *Thermus thermophilus* DNA polymerase or *Thermus aquaticus* DNA polymerase.
- 21. The method of any of claims 11 to 20, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
 - 22. The method of any of claims 11 to 21, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, P, Q, or D.

20

35

40

45



EPO - Munich 69 1 2 April 2001

PA 36450

AND ELECTIVE SERVICE CONTRACTOR C

TO ALL TO WHOM THESE; PRESENTS SHAM, COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 13, 2001

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/198,336

FILING DATE: April 18, 2000

By Authority of the COMMISSIONER OF PATENTS AND TRADEMARKS

H. PHILLIPS
Certifying Officer





TITLE OF THE INVENTION

HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES

CORRESPONDENCE ADDRESS

Customer Number 22829

ENCLOSED APPLICATION PARTS (check all that apply)

[X] (Specification - No. of Pages 37	[X] Declaration - No. of Pages5
	METHOD OF	PAYMENT
[X]	The Commissioner is hereby authorized to charge any fees or credit any overpayment to Deposit Account Number: 500812	Provisional Filing Fee Amount \$150.00
	cy of the United States Government.	States Government or under a contract with an

[] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Agent for Applicant(s)
Douglas A. Petry, Ph.D.
Reg. No. 35,321
1145 Atlantic Avenue
Alameda, CA 94501
(510) 814-2874 Telephone
(510) 814-2977 Telefax

COSTAC SCESSTOR

CERTIFICATION UNDER 37 CFR L.10

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Docket No. RPA1006P

INVENTOR(s)/APPLICANT(s)

Name: (last, first, middle) SMITH, EDWARD S.

Residence: San Francisco, California

Name: (last, first, middle) ELFSTROM, CARITA M.

Residence:

San Francisco, California

Name: (last, first, middle) GELFAND, DAVID H. Oakland, California

Residence:

Name: (last, first, middle) HIGUCHI, RUSSELL G.

Residence:

Alameda, California

Name: (last, first, middle) MYERS, THOMAS W.

Residence:

Alameda, California

Name: (last, first, middle) SCHÖNBRUNNER, NANCY J.

Residence:

Moraga, California

Name: (last, first, middle) WANG, ALICE M. Residence: Lafayette, California

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application

Inventor's Name(s): Smith et al.

Serial No.

Filed: April 18, 2000

Group:

Examiner:

For: HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES

STATEMENT UNDER 37 CFR §1.821 or §1.825

Assistant Commissioner for Patents Washingotn, D.C. 20231

Alameda, CA 94501 April 18, 2000

Sir:

I hereby certify that:

- X The contents of the paper Sequence Listing and computer readable Sequence Listing submitted herewith are the same. (37 CFR §1.821(f))
- X The contents of the paper Sequence Listing and computer readable Sequence Listing submitted herewith are the same. No new matter is included. (37 CFR §1.821(f) & (g))

- Each of the substituted sheets of the paper Sequence Listing submitted herewith is supported in the captioned application. No new matter is included. (37 CFR §1.825(a))
- The contents of the substitute paper Sequence Listing and substitute computer readable Sequence Listing submitted herewith are the same. No new matter is included. (37 CFR §1.825(a) & (b))
- The substitute computer readable Sequence Listing submitted herewith is identical to that originally-filed. (37 CFR §1.825(d))

Respectfully submitted,

Agent for Applicant(s)
Douglas A. Petry, Ph.D.

Reg. No. 35,321

1145 Atlantic Avenue

Alameda, CA 94501 (510) 814-2874 Telephone

(510) 814-2977 Telefax

. High Temperature Reverse Transcription Using Mutant DNA Polymerases

Background of the Invention

Field of the Invention

5

10

15

20

The present invention relates to the field of molecular biology and, in particular, relates to methods for the reverse transcription and amplification of ribonucleic acid (RNA) sequences.

Description of Related Art

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA polymerases. All known reverse transcriptases require a primer to synthesize a DNA transcript from an RNA template. Historically, reverse transcriptase has been used primarily to transcribe mRNA into cDNA which can then be cloned into a vector for further manipulation.

The term "DNA polymerase" describes a class of polymerases characterized as DNA-dependent DNA polymerases. DNA polymerase show a strong discrimination against using an RNA template, as expected from their functions in vivo. Nevertheless, several laboratories have shown that some DNA polymerases are capable of in vitro reverse transcription of RNA (Karkas, 1973, Proc. Nat. Acad. Sci. USA 70:3834-3838; Gulati et al., 1974, Proc. Nat. Acad. Sci. USA 71:1035-1039; and Wittig and Wittig, 1978, Nuc. Acid Res. 5:1165-1178). Gulati et al. found that *E. coli* Pol I could be used to transcribe Qβ viral RNA using oligo(dT)10 as a primer. Wittig and Wittig have shown that *E. coli* Pol I can be used to reverse transcribe tRNA that has been enzymatically elongated with oligo(dA). However, as Gulati et al. demonstrated, the amount of enzyme required and the small size of the cDNA product suggests that the reverse transcriptase activity of *E. coli* Pol I has little practical value.

T. aquaticus (Taq) DNA polymerase, a thermostable DNA polymerase, has been reported to inefficiently synthesize cDNA using Mg⁺² as the divalent metal ion (Jones and Foulkes, 1989, Nuc. Acids. Res. 176:8387-8388). Tse and Forget, 1990, Gene 88:293-296; and Shaffer et al., 1990, Anal. Biochem. 190:292-296, have described

1

25

30

methods for amplifying RNA using TapDNA-polymerase and Mg⁺² ion. However, the methods are inefficient and insensitive.

Amplification of nucleic acid sequences, both RNA and DNA, is described in U.S. Patent Nos. 4,683,195; 4,683,202; and 4,965,188; each incorporated herein by reference. A preferred method, the polymerase chain reaction (PCR), typically is carried out using a thermostable DNA polymerase, such as Taq DNA polymerase, which is able to withstand the temperatures used to denature the amplified product in each cycle. PCR is now well known in the art and has been described extensively in the scientific literature. See, for example, PCR Applications, 1999, (Innis et al., eds., Academic Press, San Diego), PCR Strategies, 1995, (Innis et al., eds., Academic Press, San Diego); PCR Protocols, 1990, (Innis et al., eds., Academic Press, San Diego); and PCR Technology, 1989, (Erlich, ed., Stockton Press, New York); each incorporated herein by reference. Commercial vendors, such as PE Biosystems (Foster City, CA) market PCR reagents and publish PCR protocols. A review of amplification methods is provided in Abramson and Myers, 1993, Current Opinion in Biotechnology 4:41-47, incorporated herein by reference.

Because reverse transcription using Taq DNA polymerase in a magnesium ion buffer was too inefficient to be practical, PCR amplification starting with an RNA template initially was carried out by first reverse-transcribing the target RNA using, for example, a non-thermostable viral reverse transcriptase such as Molony Murine Leukemia Virus Reverse Transcriptase (MoMuLV RT) or AMV-RT, and then amplifying the resulting cDNA using a thermostable DNA polymerase.

A significant advance was achieved with the discovery that a thermostable DNA polymerase could be used to efficiently reverse transcribe an RNA template by carrying out the reaction in a manganese buffer (Mn⁺²), rather than a magnesium (Mg⁺²) buffer, as is preferred for primer extension using a DNA template. Efficient Mn⁺²-activated reverse transcription using a thermostable DNA polymerase is described in U.S. Patent Nos. 5,310,652; 5,322,770; 5,407,800; 5,641,864; 5,561,058; and 5,693,517, all incorporated herein by reference. As both the synthesis of cDNA from an RNA template and the synthesis of DNA from a DNA template can be carried out in a Mn⁺² buffer, the use of a

15

20

25

30

Mn⁺² buffer enables single-enzyme, coupled reverse transcription/amplification reactions & section (see also Myers and Sigua, 1995, in PCR Strategies, supra, chapter 5).

Summary of Invention

The present invention provides methods of reverse transcribing RNA sequences using a thermostable DNA polymerase. The present invention further provides methods for reverse transcribing and amplifying RNA sequences, preferably using a single thermostable DNA polymerase in a coupled, one-tube reaction. The methods of the present invention provide improved reverse transcription ("RT") efficiency relative to previously described high-temperature reverse transcription methods.

In a preferred embodiment, the invention provides methods of reverse transcribing RNA sequences in a magnesium ion (Mg⁺²) buffer and, further, methods for reverse transcribing and amplifying RNA sequences using a single thermostable DNA polymerase in a Mg⁺² buffer, preferably in a coupled, one-tube reaction. The methods carried out using a Mg⁺² buffer provide enhanced fidelity over previously described methods that rely on manganese (Mn⁺²) activation of a thermostable DNA polymerase.

The methods of the present invention use a mutant thermoactive, preferably thermostable, DNA polymerase that contains a point mutation in a critical amino acid position previously described as affecting the DNA polymerase's ability to incorporate dideoxynucleotides (ddNTP's) labeled with fluorescein or cyanine family dyes. The present invention results from the surprising discovery that these mutant DNA polymerases also exhibit a significantly increased ability to carry out reverse transcription, particularly in a Mg⁺² buffer.

Mutant DNA polymerases useful in the methods of the present invention are described in European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496, each incorporated herein by reference. These mutant DNA polymerases are described as exhibiting an increased ability to incorporate nucleotides, including deoxynucleotides (dNTP's) and base analogues such as dideoxynucleotides (ddNTP's), labeled with fluorescein and cyanine family dyes. For convenience, these mutant DNA polymerases are referred to herein as "fluorescein family dye incorporating" DNA polymerases, or

15

"FDI? DNA polymerases. The primary utility described for FDI DNA polymerases is in DNA sequencing reactions that use dye-terminators (dye labeled ddNTP's) labeled with fluorescein or cyanine family dyes. Because a wild-type DNA polymerase discriminates against nucleotide analogues, and even more so against labeled nucleotide analogues,

dye-terminator sequencing reactions typically were carried out using an excess of dyeterminators. By decreasing the discrimination against the labeled dye-terminators, FDI DNA polymerases permit sequencing reactions to be carried out with a significantly lower concentration of dye-terminators.

The critical amino acid position that is mutated in the DNA polymerases used in the present methods, which is the same critical amino acid that affects the DNA polymerase's ability to incorporate dideoxynucleotides labeled with fluorescein and cyanine family dyes, is identified in European Patent Publication No. 0 902,035, and copending U.S. Application No. 09/146,631 by its location within a conserved sequence motif present in the native form of the enzyme. Examples of the sequence motif in a number of DNA polymerases is provided therein in Table 1. The sequence motif and the critical amino acid are identified below in essentially the same manner.

Described most generally, using the standard single-letter abbreviations for amino acids, this critical motif in the native form of the DNA polymerase comprises the amino acid sequence

20 LXXXXXXXXE (SEQ ID NO: 1),

wherein X at position 2 is S or A, X at positions 3, 4, 6, 7, 8, 9, and 10 are any amino acid, and X at position 5 is L or I.

In a more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSXELXIPYEE (SEQ ID NO: 2),

wherein X at position 3 is Q or G, and X at position 6 is S or A. Examples of DNA polymerases containing this motif are DNA polymerases from the genus *Thermus*.

In a preferred embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSQELAIPYEE (SEQ ID NO: 3).

30

25

10

15

20

25

Examples of DNA polymerases containing this motifiare DNA polymerases from Thermus species aquaticus, thermophilus, ZO5, and caldophilus.

In another preferred embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSXELSIPYEE (SEQ ID NO: 4),

wherein X at position 3 is Q or G. Examples of DNA polymerases containing this motif are DNA polymerases from *Thermus* species *flavus*, sps17, and *filiformis*.

In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSVRLGXPVKE (SEQ ID NO: 5);

wherein X at position 7 is V or I. Examples of DNA polymerases containing this motif are DNA polymerases from *Thermotoga* species maritima and neopolitana.

In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LSKRIGLSVSE (SEQ ID NO: 6).

An example of a DNA polymerase containing this motif is the DNA polymerases from Thermosipho africanus.

In another more specific embodiment, the critical motif in the native form of the DNA polymerase comprises the amino acid sequence

LAQNLNIXRKE (SEQ ID NO: 7),

wherein X at position 8 is S or T. Examples of DNA polymerases containing this motif are DNA polymerases from *Bacillus* species caldotenax and stearothermophilus.

In each of the critical motifs identified above, the critical amino acid is at amino acid position 4.

As demonstrated in the examples, mutation of the critical amino acid to any amino acid other than E (present in the native DNA polymerase used in the examples), A, G, or P provided improved RT efficiency. Thus, the methods of the present invention use a thermoactive, preferably thermostable, mutant DNA polymerase characterized in that the native form of the DNA polymerase comprises a sequence motif selected from the group consisting of SEQ ID NOs: 1 - 7, and the amino acid at position 4 of the motif is mutated to any amino acid other than E, A, G, or P. Preferably, the critical amino acid is

15

30

mutated to any amino acid other than E, A, G, P, or Q; more preferably to any amino acid other than E, A, G, P, Q, or D.

One aspect of the invention relates to methods of reverse transcribing an RNA, which comprises carrying out a reverse transcription reaction using a mutant thermoactive or thermostable DNA polymerase as described herein. Preferably, the methods comprise:

- (a) providing a reaction mixture comprising a primer sufficiently complementary to the RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to the target RNA and a mutant thermoactive or thermostable DNA polymerase as described herein; and
- (b) treating the reaction mixture under appropriate conditions to provide singlestranded cDNA.

In a preferred embodiment, the reaction of step (a) is carried out in an appropriate buffer, wherein the buffer comprises Mg^{+2} .

Another aspect of the invention relates to methods for amplifying an RNA, which comprise carrying out a single-enzyme, combined reverse transcription/amplification reaction using a mutant thermoactive or thermostable DNA polymerase as described herein. Preferably, the methods comprise:

- (a) providing a reaction mixture comprising a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to the target RNA, and the second primer is sufficiently homologous to the target RNA to hybridize to the cDNA and initiate synthesis of an extension product, and a mutant thermoactive or thermostable DNA polymerase as described herein, in an appropriate buffer, wherein the buffer comprises Mg⁺²;
 - (b) treating the reaction mixture under appropriate conditions to provide single-stranded cDNA; and
 - (c) treating the reaction mixture of step (b) under appropriate conditions to amplify the cDNA of step (b).
 - In a preferred embodiment, the reverse transcription and amplification is carried out as a single-enzyme, one-tube, coupled reverse transcription/PCR amplification

10

15

20

25

30

reaction using a mutant thermoactive or thermostable DNA polymerase as described herein in a buffer comprising Mg⁺².

Detailed Description of the Invention

To aid in understanding the invention, several terms are defined below.

The term "thermoactive DNA polymerase", as used herein, refers to a DNA polymerase that has an elevated temperature reaction optimum. The thermoactive enzyme used in the present invention catalyzes primer extension optimally at a temperature between 60 and 90°C.

The term "thermostable DNA polymerase" refers to a DNA polymerase that is stable to heat, i.e., does not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. The heating conditions necessary for nucleic acid denaturation are well known in the art. As used herein, a thermostable polymerase is suitable for use in a temperature cycling reaction such as the polymerase chain reaction ("PCR") amplification methods described in 4,965,188, incorporated herein by reference.

A "high-temperature reverse transcription reaction", as used herein, refers to a reverse transcription reaction carried out at a temperature at least 40°C, preferably, 40°C-80°C, and more preferably 50°C-70°C.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a recoverable bioactive polypeptide or precursor.

The term "native" refers to a gene or gene product which is isolated from a naturally occurring source. This term also refers to a recombinant form of the native protein produced by molecular biological techniques which has an amino acid sequence identical to that of the native form.

The term "mutant" refers to a gene that has been altered in its nucleic acid sequence or a gene product which has been altered in its amino acid sequence, resulting in a gene product which may have altered functional properties when compared to the native or wild-type gene or gene product. Such alterations include point mutations, deletions and insertions.

10

15

20

25

30

in As-used herein, a "point mutation" in an amino acid sequence refers to either a single amino acid substitution or single amino acid deletion. A point mutation preferably is introduced into an amino acid sequence by a suitable codon change in the encoding DNA.

Individual amino acids in a sequence are represented herein as AN, wherein A is the amino acid in the sequence and N is the position in the sequence. Substitution-type point mutations within an amino acid sequence are represented herein as A₁NA₂, wherein A₁ is the amino acid in the unmutated protein sequence, A₂ is the amino acid in the mutated protein sequence, and N is the position in the amino acid sequence. Either the one-letter or three-letter codes are used for designating amino acids (see Lehninger, BioChemistry 2nd ed., 1975, Worth Publishers, Inc. New York, NY: pages 73-75, incorporated herein by reference). For example, a G46D mutation represents a change from glycine to aspartic acid at amino acid position 46. The amino acid positions are numbered based on the full-length sequence of the protein from which the region encompassing the mutation is derived. Representations of nucleotides and point mutations in DNA sequences are analogous.

The terms "nucleic acid" and "oligonucleotide" refer to primers, probes, and oligomer fragments to be detected, and shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. There is no intended distinction in length between the terms "nucleic acid" and "oligonucleotide", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA.

Oligonucleotides can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang et al., 1979, Meth. Enzymol. 68:90-99; the phosphodiester method of Brown et al., 1979, Meth. Enzymol. 68:109-151; the diethylphosphoramidite method of Beaucage et al., 1981, Tetrahedron Lett. 22:1859-1862; and the solid support method of U.S. Patent No. 4,458,066, each incorporated

10

15

30

herein by reference. Automated synthesis using cyanoethyl phosphoramidite chemistry is preferred. Reagents and instruments are commercially available from, for example, PE Biosystems (Foster City, CA) and Pharmacia (Piscataway, NJ).

The term "primer", as used herein, refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. A primer is preferably a single-stranded oligodeoxyribonucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 35 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template for primer elongation to occur.

A "pair of primers", as used herein, refers to a first and second primer selected to function in an amplification reaction, such as a polymerase chain reaction, to amplify a desired target sequence. For example, for use in a coupled reverse transcription/amplification reaction to amplify a target RNA, a pair of primers comprises a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA molecule complementary to the target RNA, and said second primer is sufficiently homologous to said target RNA to hybridize to the cDNA and initiate synthesis of an extension product. The design of primer pairs for the amplification of nucleic acid sequences is well known in the art.

A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ³²P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in ELISA assays), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available.

As used herein the term "cDNA" refers to a copy DNA molecule synthesized using a ribonucleic acid strand (RNA) as a template. The RNA may be mRNA, tRNA, rRNA, or another form of RNA, such as viral RNA. The cDNA may be single-stranded,

..double-stranded or may be hydrogen-bonded to a complementary RNA molecule as in an RNA/cDNA hybrid.

The term "reverse transcription reaction mixture" refers to an aqueous solution comprising the various reagents used to reverse transcribe a target RNA. These include enzymes, aqueous buffers, salts, oligonucleotide primers, target nucleic acid, and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete reverse transcription reaction mixture.

The term "amplification reaction mixture" refers to an aqueous solution comprising the various reagents used to amplify a target nucleic acid. These include enzymes, aqueous buffers, salts, amplification primers, target nucleic acid, and nucleoside triphosphates. Depending upon the context, the mixture can be either a complete or incomplete amplification reaction mixture. In the preferred embodiment of the invention, the amplification reaction is a polymerase chain reaction (PCR) and the amplification reaction mixture is a PCR mixture. As used herein, an amplification reaction mixture encompasses the reaction mixture used for the amplification of an RNA, as in a coupled reverse transcription/amplification reaction.

The term "buffer," as used herein, refers to a solution containing a buffering agent or a mixture of buffering agents and, optionally, a divalent cation and a monovalent cation.

Conventional techniques of molecular biology and nucleic acid chemistry, which are within the skill of the art, are fully explained in the literature. See, for example, Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Nucleic Acid Hybridization (B.D. Hames and S.J. Higgins. eds., 1984); Basic Methods in Molecular Biology (Elsevier, NY); Current Protocols in Molecular Biology (John Wiley and Sons, NY); and a series, Methods in Enzymology (Academic Press, Inc.), all of which are incorporated herein by reference. All patents, patent applications, and publications mentioned herein, both supra and infra, are incorporated herein by reference.

30

25

15

20

Mutant DNA Polymerases

The mutant DNA polymerases used in the methods of the present invention contain a point mutation at a critical amino acid position identified in European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496, each incorporated herein by reference. European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631 identify the critical amino acid in terms of its position within a conserved critical sequence motif found in the native DNA polymerase sequence. PCT International Patent Publication No. WO 98/40496 identifies the critical amino acid in Taq DNA polymerase by position number (E681) and in other DNA polymerase by amino acid sequence homology to Taq DNA polymerase. Both methods identify the same critical amino acid position. For clarity of description, the critical amino acid is described herein in terms of its position in the conserved critical sequence motif.

Table 1, reproduced in essence from Table 1 of European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631, provides the critical motifs found in a number of representative DNA polymerases (the critical amino acid is highlighted) along with positions of the critical amino acid within the full enzyme sequence. Multiple position numbers are provided where slightly different amino acid sequences for DNA polymerases from the same species have been reported in the literature.

30

35

PATENT Atty. Docket No. RPA1006P

Table	1

		SEO ID NO:	Critical Motif	Position
	Thermus			
5	aquaticus	8	LSQBLAIPYEE	681
	flavus	9	LSGELSIPYEE	679
	thermophilus	10	LSQKLAIPYEE	683
	species Z05	11	LSQELAIPYEE	683
	species sps 17	12	LSQELSIPYEE	679
10	caldophilus	13	LSQBLAIPYEE	683
	filiformis	14	LSQELSIPYEE	679
	Thermotoga		-	
	maritima	15	LSVRLGVPVKE	744
	neapolitana	16	LSVRLGIPVKE	744
15	Thermosipho			
	africanus	17	LSKRIGLSVSE	743
	Bacillus			
	caldotenax	18	LAQNLNISRKE	725, 724
	stearothermoph	ilus 19	LAQNLNITRKE	724, 727, 802
20	•			

A number of species possessing a thermoactive DNA polymerase having the critical motif are described in European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496. Preferred DNA polymerases for use in the present invention are derived from a *Thermus* species.

The mutant DNA polymerase used in the methods of the present invention is a thermoactive, preferably thermostable, mutant DNA polymerase characterized in that the native form of the DNA polymerase comprises a sequence motif selected from the group consisting of SEQ ID NOs: 1-7, and the amino acid at position 4 of the motif is mutated to any amino acid other than E, A, G, or P. Preferably, the critical amino acid is mutated to any amino acid other than E, A, G, P, or Q, more preferably to any amino acid other than E, A, G, P, Q, or D.

The mutant DNA polymerase can be derived from any species possessing a thermoactive DNA polymerase having the critical motif in the polymerase domain. The critical motif identifies a particular functional region within the polymerase domain of the enzyme, and identifies an amino acid within the motif that is critical to the function. The

15

20

25

30

examples describe the effects on Mg⁺²-activated reverse transcription efficiency of each possible mutation at this site in one widely used thermostable DNA polymerase, *Thermus thermophilus* DNA polymerase. Just as amino acid changes at this site affect the efficiency of incorporation of dideoxynucleotides (ddNTP's) labeled with fluorescein or cyanine family dyes in essentially all DNA polymerases having the conserved critical motif, it is expected that amino acid changes at this site will affect the Mg⁺²-activated reverse transcription efficiency of essentially all DNA polymerases having the conserved critical motif.

The structural relatedness of DNA polymerases and the presence of conserved functional domains is well known (see, for example, Ito and Braithwaite, 1991, Nucl. Acids Res. 19(15):4045-4-47; Blanco et al. 1991, Gene 100:27-38; Gutman and Minton, 1993, Nucl. Acids. Res. 21(18):4406-4407; and Delarue et al., 1990, Protein Engineering 3(6):461-467; each incorporated herein by reference). Mutations of a critical amino acid within a conserved functional domain, in general, are expected to have analogous effects when introduced into other DNA polymerases (see, for example, Xu et al., 1997, J. Mol. Biol. 268:284-302; U.S. Patent Nos. 5,466,591; 5,795,762; 5,939,292; and 5,614,365; each incorporated herein by reference).

Additional thermoactive or thermostable DNA polymerases containing the critical motif, and the position of the critical amino acid therein, can be identified routinely by direct inspection of the amino acid sequence. Additionally, the critical motif and amino acid can be identified by sequence homology with another DNA polymerase known to contain the critical motif, such as the DNA polymerases from the *Thermus* species listed in Table 1. Amino acid and nucleic acid sequence alignment programs are readily available. For example, widely used sequence alignment programs, including "GAP," "BESTFIT," and "PILEUP," are available from the Genetics Computer Group (Madison, Wisconsin). In general, carrying out a sequence alignment using the default parameters facilitates identification of the critical amino acid in a DNA polymerase sequence homologous to the critical amino in one of the DNA polymerases listed in Table 1.

As new DNA polymerase sequences are obtained, sequences may be discovered that contain a variant of the critical motif that is not literally described by SEQ ID NO: 1, but is identifiable by sequence homology with the known enzymes. As a hypothetical

Exexample, an enzyme having a motif in the DNA polymerase domain that is a variant of SEQ ID NO: 3, differing only in that the amino acid at position 5 is other than L or I, would be recognized as having the critical motif in view of the high homology (10 out of 11 amino acids in this example) with the critical motif of several *Thermus* species enzymes. Such an enzyme is considered equivalent for the purposes of the present invention.

The critical amino acid is identified with reference to the native enzyme. However, this is not meant to restrict the amino acid sequence of the mutant enzyme everywhere else to that of the native enzyme. Mutant DNA polymerases used in the methods of the present invention may contain additional mutations whose presence may be advantageous for particular applications. For example, mutations which eliminate 5' to 3' exonuclease activity or 3' to 5' exonuclease activity and their applications are well known. An additional substitution mutation in position 3 of the critical motifs identified as SEQ ID NOs: 1-7 (for example, a Q682K, E683K mutant of Tth DNA polymerase) may provide additional benefits, particularly in Mn⁺²-activated reactions, such as allowing a further reduction of the Mn⁺² concentration or further broadening the range of usable salt concentrations.

Mutant DNA polymerases for use in the methods of present inventions preferably are expressed from recombinant expression vectors in which the coding sequence has been modified to express the particular mutant protein sequence of interest. Methods for introducing point mutations into a coding sequence in an expression plasmid are well known in the art and described in the patent and scientific literature incorporated herein by reference. Detailed protocols are provided in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989, second edition, chapter 15.51, "Oligonucleotide-mediated mutagenesis," and Ausebel et al., Current Protocols in Molecular Biology (current edition), both of which are incorporated herein by reference. European Patent Publication No. 0 902,035, co-pending U.S. Application No. 09/146,631, and PCT International Patent Publication No. WO 98/40496 teach the construction of appropriate expression vectors and the expression and purification of the resulting mutant DNA polymerase. Following the guidance provided in the cited references, and using only well known techniques, one skilled in the art will be able to

prepare any number of expression vectors containing a mutant gene suitable for expressing in any of a variety of host systems mutant DNA polymerases for use in the methods of the present invention.

For use in the present high temperature reverse transcription methods, it is only essential that the DNA polymerase is thermoactive. Because the preparation of cDNA from an RNA template does not involve repeated denaturation cycles at elevated temperatures, it is not essential that enzymes useful in the method are thermostable as well as thermoactive. In the single enzyme, combined reverse transcription/polymerase chain reaction amplification (RT/PCR) methods described in the examples, use of a thermostable DNA polymerase is preferred because the DNA polymerase is subject both to RT conditions and to the PCR conditions, which included repeated denaturation cycles.

Divalent Cation

5

10

15

20

25

For reverse transcription, according to the present invention, the reaction is carried out in a mixture containing the RNA template, a primer, and a thermoactive or thermostable mutant DNA polymerase. The reaction mixture typically contains all four deoxyribonucleotide triphosphates (dNTPs) and a buffer containing a divalent cation and a monovalent cation. DNA polymerases require a divalent cation for catalytic activity. For extension reactions using a DNA template, the preferred divalent cation is Mg⁺², although other cations, such as Mn⁺² or Co⁺² can activate DNA polymerases.

In contrast to extension reactions using a thermoactive or thermostable DNA polymerase and a DNA template, extension reactions using an RNA template, i.e., reverse-transcription, essentially have required the use of Mn⁺² in order to achieve useful efficiency. For example, the use of MnCl₂ or Mn(OAc)₂ for RNA amplification with Tth DNA polymerase provides an increase in sensitivity of at least 10⁶-fold compared to the use of MgCl₂ and standard PCR conditions.

The use of Mn⁺², although it increases the efficiency of reverse transcription, also decreases the fidelity, resulting in an increased number of misincorporated nucleotides. The use of Mn⁺² also decreases the fidelity of DNA amplifications. Thus, single-enzyme, one-tube RNA amplification reactions using Mn⁺² suffer reduced fidelity in both the RNA and DNA phases of the reaction. Thus, when higher fidelity RNA amplification is

20

25

30

desired, it is preferable to carry out the reaction in two stages. This is achieved by effectively removing the Mn⁺² ions from the reverse-transcription mixture using a chelator, such as EDTA or, preferably, EGTA, and then adding an appropriate Mg⁺²-containing DNA amplification mixture to complete the reaction.

The use of the mutant DNA polymerases in the methods of the present invention provide benefits for reverse transcription reactions regardless of the divalent cation used. In Mn⁺² reactions, the use of the mutant DNA polymerase provides for high temperature reverse-transcription and amplification of RNA with a higher efficiency than achieved using the native enzyme. In addition, the use of the mutant DNA polymerase allows carrying out the reaction at a lower Mn⁺² concentration, thereby minimizing the deleterious effect of Mn⁺² concentration on fidelity.

Particularly surprising is that the mutant DNA polymerases enable reverse transcription to be carried out using Mg⁺² with significantly increased efficiency. The use of Mg⁺², the enzyme's preferred divalent cation, provides for significantly higher fidelity. Thus, in Mg⁺² reactions, the use of the mutant DNA polymerase provides for high temperature, high fidelity reverse-transcription and amplification of RNA with a usable efficiency.

The divalent cation is supplied in the form of a salt such MgCl₂, Mg(OAc)₂, MgSO₄, MnCl₂, Mn(OAc)₂, or MnSO₄. In general, for reactions using Mn⁺², usable cation concentrations in a Tris-HCl buffer will be in a range from 0.5 to 7 mM MnCl₂, preferably between 0.5 and 2 mM, and in a bicine/KOAc buffer or tricine/KOAc buffer will be in a range from 0.5 to 20 mM Mn(OAc)₂, preferably between 0.5 and 5 mM. In general, for reactions using Mg⁺², usable divalent cation concentrations in a Tris-HCl buffer will be in a range from 0.5 to 10 mM MgCl₂, and in a bicine/KOAc or tricine/KOAC buffers, will be in a range from 0.5 to 20 mM for Mg(OAc)₂, preferably between 0.5 and 5 mM. These concentrations provide useful starting conditions for carrying out routine reaction optimization. The optimal divalent ion concentration in a particular reaction will depend not only on the particular enzyme used, but also on the other reaction components, such as, for example, the dNTP concentration and primer sequence and concentration. One of skill will understand that reaction conditions in

20

25

30

for any particular reaction using routine experimental methods.

Previously, while capable of activating RNA template-directed DNA synthesis, mixed divalent cation buffers (e.g., Mn⁺² and Mg⁺²), were not preferred due to reduced sensitivity and efficiency. It is expected that mixed divalent cation buffers are useful in the methods of the present invention and, in some applications, may be preferable. Mixed cation use may enable, for example, a tradeoff between a higher efficiency, but lower fidelity Mn⁺²-activated reaction and a higher fidelity Mg⁺²-activated reaction.

10 Reverse Transcription and Amplification Methods

High temperature reverse transcription methods and combined reverse transcription/amplification methods using a thermostable DNA polymerase in a Mn⁺² buffer are well known in the art. See, for example, U.S. Patent Nos. 5,310,652; 5,322,770; 5,407,800; 5,561,058; 5,641,864; and 5,693,517; each incorporated herein by reference. The methods of the present invention represent a modification of the previously described methods, wherein the modification involves the use of mutant DNA polymerases, as described above. In a preferred embodiment, the reaction is carried out in buffer containing Mg⁺² as the divalent cation used to activate the DNA polymerase.

One advantage of the present methods is that the use of the mutant DNA polymerases appears to provide faster RT extension rates and, consequently, less time is needed for the RT reaction. Preferably, to maximize the amount of cDNA produced in a reverse transcription reaction, the reaction is carried out for about 30 minutes.

Depending on the application, particularly in manganese reactions, RT times as short as one minute or less may provide acceptable results.

Other advantages of the present methods are that the use of the mutant DNA polymerases may provide improved RT efficiency at lower enzyme concentrations and, furthermore, provide a wider range of usable salt concentrations. It is expected that optimal reaction conditions will depend on, for example, the particular enzyme used and can be determined empirically in a routine manner.

Other aspects required to carry out the present methods, such as selection of a target RNA, sample preparation, primer design, and choice of reagents and reaction

"conditions other than the DNA polymerase and divalent cation used to activate the DNA polymerase are well known in the art and described in, for example, the above-referenced patents. Similarly, if the reverse transcription is coupled with an amplification reaction, all aspects of the amplification not relating to the DNA polymerase and divalent cation used to activate the DNA polymerase are well known in the art and described in, for example, the above-referenced patents. Finally, applications of reverse transcription and amplification of RNA are well known in the art and described in, for example, the above-referenced patents. One of skill in the art will be able to apply the present methods in any application in which the reverse transcription and, optionally, amplification of RNA is desired.

The following examples are offered by way of illustration only and should not be construed as intending to limit the invention in any manner.

Example 1

15

20

30

10

Examples of Mutant DNA polymerase

A series of 19 mutant DNA polymerases were constructed from "native" Thermus thermophilus (Tth) DNA polymerase representing all possible mutations in the critical amino acid. As described in European Patent Publication No. 0 902,035 and co-pending U.S. Application No. 09/146,631, Tth DNA polymerase amino acid sequence contains the critical sequence motif represented as SEQ ID NO: 3 (which is a particular embodiment of SEQ ID NO: 2, which is a narrower embodiment of the general motif, SEQ ID NO: 1). The critical amino acid is at position 683 (E683).

The sequence of Tth DNA polymerase and plasmids containing the gene for Tth DNA polymerase are known in the art (see, for example, U.S. Patent Nos. 5,618,711 and 5,789,224, both incorporated herein by reference). The particular plasmid used in the present example encodes a Tth DNA polymerase also containing a G46B point mutation that eliminates the 5' to 3' exonuclease activity of the enzyme, as described in U.S. Patent No. 5,466,591, incorporated herein by reference. In addition, the plasmid contains silent nucleotide substitutions that introduce a *ClaI* recognition and cleavage site encompassing codons 678, 679, and the first nucleotide of 680, without changing the encoded amino acid sequence. The presence of the additional mutation in the 5' to 3'

exonuclease domain is believed to have no appreciable effect on the ability of the DNA polymerase to reverse transcribe RNA in a Mg⁺² buffer; Tth DNA polymerase having the G46E mutation is considered herein as the native DNA polymerase.

Point mutations in the expressed proteins were introduced by mutating the encoding DNA sequence using standard techniques. Essentially, a short fragment of the coding sequence encompassing codon 683 was replaced with a synthetic fragment containing the desired sequence. The short fragment, ~65 nucleotides in length, was excised by digesting the plasmid with restriction enzymes ClaI and HindIII. A synthetic double-stranded DNA insert was synthesized encoding the same amino acid sequence as the excised fragment, but containing the desired mutation in codon 683. The synthetic fragment was then ligated into the digested plasmid, yielding a plasmid containing a mutated codon encoding a full length Tth DNA polymerase having the desired point mutation.

15

20

25

30

10

Example 2

Reverse Transcription/Amplification Efficiency

The 20 DNA polymerases described in Example 1 (1 native and 19 mutants) were compared for their ability to catalyze reverse transcription/amplification reactions. In overview, coupled, single-enzyme reverse transcription/amplification reactions were carried out with each of the DNA polymerases. The same initial target copy number was used for each reaction, and the synthesis of amplification product was monitored during the reaction. The number of cycles required to generate an arbitrary, but fixed, quantity of amplified product, which provides a measure of the reaction efficiency, was determined for each DNA polymerase. Because the initial reverse transcription step typically is the critical limiting step in a reverse transcription/amplification reaction, an improvement in overall reaction efficiency also suggests an improvement in the initial reverse transcription step.

The increase in amplified nucleic acid during the reaction was monitored using the methods described in Higuchi et al., 1992, <u>Bio/Technology 10</u>:413-417; Higuchi et al., 1993, <u>Bio/Technology 11</u>:1026-1030; Higuchi and Watson, in PCR Applications, supra, Chapter 16; U.S. Patent No. 5,994,056; and European Patent Publication Nos.

487,218 and 512,334, each incorporated herein by reference. These methods, referred to herein as kinetic-PCR, rely on the increased fluorescence that ethidium bromide (EtBr) and other DNA-binding dyes exhibit when bound to double-stranded DNA in order to detect the change in amount of double-stranded DNA in a reaction. The increase of double-stranded DNA resulting from the synthesis of target sequences results in an increase in the amount of dye bound to double-stranded DNA and a concomitant detectable increase in fluorescence.

Amplifications were carried out with ethidium bromide in the reaction.

Alternatively, amplifications can be carried out using SYBR® Green I (Molecular Probes, Eugene, OR) in the reaction. Both dyes increase their fluorescence upon intercalation into, or binding to, the double-stranded DNA. The reactions are carried out in a combined thermal cycler and fluorescence detection system which enables monitoring the fluorescence of the reaction mixture during the amplification. It will be clear that, in addition to the instruments described below, any suitable instrument can be used.

15

20

25

10

RNA Target and Amplification Primers

A target RNA was synthsized using an expression plasmid encoding the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. A region of the GAPDH RNA was amplified using the following primers, shown in the 5' to 3' orientation:

P1 (SEQ ID NO: 20) 5'-CGAGATCCCTCCAAAATCAA
P2 (SEQ ID NO: 21) 5'-CATGAGTCCTTCCACGATACCAA

The initial target concentration was measured by standard means. For the comparison reactions described herein, the absolute copy number is less important than the relative copy number. To insure the same initial copy number in each reaction, aliquots of dilutions of the same initial RNA stock were used.

One of skill will recognize the selection of target is a matter of convenience.

Other RNA targets and corresponding amplification primers could be used in essentially the same protocol. Routine optimization of the reaction conditions would be expected.

25

30

Amplification

Each RT-PCR amplification was carried out in a total reaction volume of 100 μ l. The final reagent concentrations were as follows:

10 units DNA polymerase

10⁶ copies GAPDH RNA
50 mM Tricine, pH 8.15
50 mM KOAc
2 mM Mg(OAc)₂
200 µM dATP, dGTP, dCTP
400 µM dUTP
200 nM each primer
8% glycerol
1% DMSO
1 µg/ml of ethidium bromide
15 2 units UNG*

* Manufactured by Roche Molecular Systems and commercially available through PE Biosystems, Foster City, CA.

Alternatively, SYBR® Green I can be used instead of ethidium bromide to enable detection of the amplified product. Amplifications using SYBR® Green I are carried out with 0.2 X SYBR® Green I (sold as 10,000 X) diluted in DMSO.

Reactions using ethidium bromide preferably are carried out using a ABI PRISM® 7700 Sequence Detection System (PE Biosystems, Foster City, CA), which allows the selection of suitable detection wavelengths. Reactions using SYBR® Green I preferably are carried out using a GeneAmp® 5700 Sequence Detection System (PE Biosystems, Foster City, CA) using the same thermal cycling conditions. The GeneAmp® 5700 Sequence Detection System is designed for use with SYBR® Green I and the excitation and detection wavelengths are pre-set for this dye.

The assays described below were carried out using a custom instrument consisting essentially of a GeneAmp® PCR system 9600 thermal cycler (PE Biosystems, Foster City, CA) modified by the addition of a fluoresence detection system similar to that used in the GeneAmp® 5700 Sequence Detection System, but designed for use with ethidium bromide. Results obtained using the custom instrument would be expected to be comparable to results obtained using one of the preferred instruments.

Amplification reactions were carried out using the specific temperature cycling • 35 profile shown below.

Thermal Cycling Times and Temperatures

Pre-reaction incubation:

50°C for 2 minutes

Reverse-transcription

60°C for 30 minutes 95°C for 1 minute

55 cycles:

denature:

95°C for 15 seconds

anneal: extend: 55°C for 30 seconds 72°C for 15 seconds

Final extension and hold:

72°C

Detection

The accumulation of amplified product was measured at each cycle during the reaction by measuring the increase in reaction fluorescence. During each amplification cycle, each reaction was excited with light at a wavelength near the excitation maximum of the dye and the emission of the dye was measured near its emission maximum.

Fluorescence measurements were normalized by dividing by an initial fluorescence measurement obtained during a cycle early in the reaction while the fluorescence measurements between cycles appear to be relatively constant. The cycle number chosen for the initial fluorescence measurement was the same for all reactions compared, so that all measurements represent increases relative to the same reaction cycle.

The number of amplification cycles carried out until the fluorescence exceeded an arbitrary fluorescence level (AFL) was calculated from the observed fluorescence values. The AFL was chosen close to the baseline fluorescence level, but above the range of random fluctuations in the measured fluorescence, so that the reaction kinetics were measured during the early phase of the amplification when the amount of product increases geometrically. During this geometric growth phase of the amplification, the number of cycles required to reach a particular threshold value depends solely on the initial copy number and the reaction efficiency. As each reaction is carried out using the same initial target copy number, the number of cycles to reach the threshold provides a

measure of the reaction efficiency. In later cycles, accumulation of amplified product and exhaustion of reagents eventually leads to a reaction plateau.

An AFL of 1.12 was chosen for all reactions. Because a PCR amplification consists of discrete cycles and the fluorescence measurements are carried out once per cycle, the measured fluorescence typically increases from below the AFL to above the AFL in a single cycle. To improve the precision of the measurements, an "exact" number of cycles to reach the AFL threshold, referred to herein as the C_T value, was calculated by interpolating fluorescence measurements between cycles.

10 Results:

15

The C_T values obtained using the native Tth DNA polymerase (E683) and each of the mutant DNA polymerases (identified by amino acid at position 683) are shown in the table, below. Each C_T value represents the average value obtained from two reactions. To facilitate comparison, the difference of the C_T values, (C_T native) - (C_T mutant), also is provided. An increase in efficiency using the mutant DNA polymerase results in reaching the threshold value in fewer cycles, i.e., a lower C_T value. Thus, a positive difference in C_T values reflects an increase in efficiency.

Reaction Efficiencies

аа @ 683	aa @ 683	Ave. CT	(C _T native) - (C _T mutant)
1-letter	3-letter	[
code	code	<u> </u>	
В	Glu	37.7	0.0
Α	Ala	38.7:	-1.1
С	Cys	33.2	4.5
D	Asp	36.6	1.1
F	Phe	28.2	9.5
G	Gly	42.3	-4.7
H	His	32.0	5.7
I	Ile	33.4	4.3
K	Lys	27.6	10.1
L	Leu	27.8	9.9
M	Met	30.3	7.4
N	Asn	31.5	6.2
P	Pro	38.8	-1.1
Q	Gln	37.4	0.3
R	Arg	27.2	10.5
S	Ser	32.0	5.7
T_	Thr	31.5	6.2
V	Val	31.4	6.3
W	Trp	29.2	8.5
Y	Туг	26.1	11.6

The results indicate that mutations of the amino acid at position 683 to any amino acid except Ala, Gly, or Pro resulted in an DNA polymerase with increased efficiency.

Of these, all but the Asp and Gln mutants resulted in a least a four cycle improvement in the C_T value.

Example 3

10

15

Reverse Transcription Efficiency

Selected DNA polymerases described in Example 1 were compared for their ability to catalyze reverse transcription reactions. Reverse transcription reactions were carried out with each of the DNA polymerases using either Mg⁺² or Mn⁺². The resulting cDNA from each of the reactions then was amplified under identical conditions using the native enzyme and Mg⁺². This protocol allows measuring the effect of the enzyme

specifically on the reverse transcription portion of a reverse transcription/amplification reaction.

In addition to the native enzyme, reactions were carried out using DNA polymerases having mutations to F, K, L, R, and Y at amino acid position 683. Each of these mutations was shown in Example 2 to provide significant increases in efficiency in a combined reverse transcription/amplification reaction.

Reverse transcription

Each reverse transcription was carried out in a total reaction volume of 100 μl.

10 The final reagent concentrations were as follows:

5 units DNA polymerase
10⁶ copies GAPDH RNA
50 mM Tricine, pH 8.15
50 mM KOAc
2 mM Mg(OAc)₂ or Mn(OAc)₂
200 μM dATP, dGTP, dCTP, dUTP
200 nM each primer
8% glycerol
1% DMSO

20 0.2 X SYBR® Green I 1 unit UNG*

* Manufactured by Roche Molecular Systems and commercially available through PE Biosystems, Foster City, CA.

The reverse trancription reactions were carried out using the specific temperature cycling profile used is shown below.

Reverse Transcription Times and Temperatures

Pre-reaction incubation:

50°C for 2 minutes

Reverse-transcription

60°C for 30 minutes

Hold:

4°C

Amplification

Following reverse transcription, 10 µl of the reaction products were added to 10 µl 2 mM EGTA to chelate the metal cation, thereby effectively removing it from the following amplification reaction. The mixture was added to a PCR amplification mixture containing the native enzyme and Mg⁺². Thus, residual mutant DNA polymerase was

diluted such that any effects were expected to be negligible. The PCR amplifications were carried out in 100 µl reactions with the following final reagent concentrations:

5 units native DNA polymerase
50 mM Tricine, pH 8.15
5 50 mM KOAc
2 mM Mg(OAc)₂
200 µM dATP, dGTP, dCTP, dUTP
200 nM each primer
8% glycerol
10 1% DMSO
0.2 X SYBR® Green I

Amplification reactions were carried out using the specific temperature cycling profile shown below.

Amplification Thermal Cycling Times and Temperatures

95°C for 1 minute

55 cycles:

15

20

denature: anneal;

95°C for 15 seconds

extend:

55°C for 30 seconds 72°C for 15 seconds

Final extension and hold:

72°C

Results

The C_T values obtained using the native Tth DNA polymerase (E683) and each of the mutant DNA polymerases (identified by amino acid at position 683) for the reverse transcription, and the native Tth DNA polymerase for all amplifications are shown in the table, below. Each C_T value represents the average value obtained from two reactions. To facilitate comparison, the difference of the C_T values, (C_T native) - (C_T mutant), also is provided. An increase in efficiency using the mutant DNA polymerase results in reaching the threshold value in fewer cycles, i.e., a lower C_T value. Thus, a positive difference in C_T values reflects an increase in efficiency.

Reaction Efficiencies

Mg⁺²-activated RT

aa @ 683	Ave. C _T	(C _T native) - (C _T mutant)
_ E	33.8	0.0
·F	26.9	. 6.9
K	29.0	4.8
L	26.0	7.8
: R	25.9	7.9
Y	24.5	. 9.3

Reaction Efficiencies

Mn⁺²-activated RT

aa @ 683	Ave. CT	(C _T native) - (C _T mutant)
E	24.6	0.0
F	21.1	3.5
K	20.2	4.4
L	20.6	4.0
R	20.7	3.9
Y	20.3	4.3

5

Each of these mutant DNA polymerase provided a significantly increased efficiency in reverse transcription/amplification reactions. Because the DNA amplification portion of each reaction was carried out identically with the native enzyme, these results demonstrate that each of these mutant DNA polymerase provide increased efficiency in the reverse transcription portion of the reactions. The mutant DNA polymerases provided significantly improved efficiency using either cation.

۲

The improvement was particularly pronounced using Mg^{+2} and demonstrates that the use of the mutant DNA polymerases makes Mg^{+2} -activated reactions practical. Consistent with what has been reported previously, RNA amplification using the native enzyme essentially requires the use of Mn^{+2} to achieve a usable reaction efficiency, as seen by the almost 10 cycle delay (33.8-24.6) in the C_T using Mg^{+2} . In contrast, using the E683Y mutant, for example, the efficiency of the Mg^{+2} -activated reaction was equal to that achieved using the native enzyme and Mn^{+2} .

10

15

20

Example 4

Fidelity

The fidelities of selected mutant DNA polymerases and the native enzyme were compared in several ways. The fidelities in coupled reverse-transcription/amplification reactions carried out in a Mg⁺² buffer were compared to the fidelities when carried out in a Mn⁺² buffer. Additionally, the fidelities of the enzymes when used in DNA amplifications were compared.

The fidelities of DNA polymerases can be compared by measuring the melting temperature (Tm) profile of amplified products generated using the enzymes. The fidelity of a DNA polymerase is reflected in the number of misincorporations occurring during strand synthesis. An amplification using a lower fidelity enzyme will result in greater heterogeneity in the resulting population of amplified sequences. To measure the heterogeneity, the amplified products are denatured, allowed to reanneal, and the Tm of the resulting duplexes is measured. Because the strands in the duplexes are combined at random from a heterogeneous population of sequences, the duplexes, in general, contain a number of mismatches. The greater the sequence heterogeneity in the population of amplified products, the greater the average number of mismatches in the duplexes. These mismatches destabilize the duplexes and result in a lower measured Tm.

A melting curve for the amplified products of a kinetic-PCR reaction is carried out conveniently using the thermal cycler/fluorescence detection instrument used in the amplification. After amplification, the relationship between fluorescence and temperature is measured over a temperature range encompassing the denaturation temperature of the product. The transition between double-stranded and single-stranded molecules is reflected in a change in dye fluorescence. Thus, a melting curve can be determined conveniently. Alternatively, measurements can be carried out using standard methods, which typically involve monitoring the change in optical density, a measure of the amount of double-stranded DNA in the reaction, with a change in temperature.

The fidelity of the native DNA polymerase was compared to the fidelities of two of the mutants, DNA polymerases containing the E683K and E683N mutations, respectively. Coupled reverse transcription/amplification reactions were carried out in duplicate in both Mn⁺² and Mg⁺² buffers essentially as described in Example 2, but with

the following changes. For the Mn⁺² reactions, 2 mM Mn(OAc)₂ was used in the reaction. All reactions were carried out using 25 U of DNA polymerase.

To measure the hybridization stability profile (melting curve) of the amplified double-stranded target sequences, the fluorescence of the post-amplification reaction mixture was monitored over a range of temperatures covering at least 60°C to 80°C. As expected, the fluorescence measurements resulted in a sigmoidal melting curve. A Tm was defined as the temperature of the inflection point in the sigmoidal melting curve, which corresponds to the temperature at which half the target is in single-stranded form.

10 Results

The Tm values measured for the amplification products of the Mg⁺²-activated reactions and the Mn⁺²-activated reactions are shown in the table, below. Each measurement reported is the average of replicate reactions. All temperature are degrees Celsius.

15

20

25

Amplification Product Tm Values

DNA Polymerase	Tm, Mg ⁺²	Tm, Mn ⁺²
Native	80 ·	78
E683K	80	76
E683N	80	76

Using a Mg⁺² buffer, no difference in fidelity was observed between the native and the mutant DNA polymerases. Similar reactions carried out using all 20 DNA polymerases (data not shown) confirmed that the fidelity of all the mutant DNA polymerases are identical to the fidelity of the native DNA polymerase in Mg⁺²-activated reactions.

Comparing the results obtained using a Mn⁺² buffer to those obtained using a Mg⁺² buffer, the fidelities of the all of the DNA polymerase was reduced, as can be seen from the lower Tm values obtained. Interestingly, the two mutant DNA polymerases exhibited an even lower fidelity than did the native enzyme when using a Mn⁺² buffer, at least under these reaction conditions.

20

25

concentration on the fidelity of the mutant and native enzymes, additional experiments were carried out using the E683K mutation using a range of Mn⁺² concentrations from 0.5 to 5 mM. The results (data not shown) showed that, as expected the lowest Mn⁺² concentration yielded the highest fidelity reactions using either enzyme. The fidelity of the mutant enzyme was more affected by an increased Mn⁺² concentration than was the fidelity of the native enzyme. Surprisingly, however, at least in these experiments, the mutant enzyme also was most efficient at the lowest Mn⁺² concentration. Thus, the use of the mutant enzyme allows carrying out the reaction at a lower Mn⁺² concentration, thereby minimizing the deleterious effect of Mn⁺² concentration on fidelity.

In addition, both Mn⁺²-activated and Mg⁺²-activated reactions also were carried out essentially as described above, but using DNA templates, which facilitates observing the effect of fidelity in only the DNA portion of the reaction. In all cases, the Tm value of the product of the DNA amplification was indistinguishable from the Tm value of the product of the RNA amplification.

The results, taken together with the results of the previous examples, demonstrate advantages of the methods of the present invention. Previously described high temperature reverse-transcription and amplification methods were carried out using Mn⁺² to achieve adequate reaction efficiency, but suffered from a reduction in fidelity. The present invention provides several options. Using Mn⁺², the use of the mutant enzyme provides for high temperature reverse-transcription and amplification of RNA with a higher efficiency than achieved using the native enzyme, and allows carrying out the reaction at a lower Mn⁺² concentration, thereby minimizing the deleterious effect of Mn⁺² concentration on fidelity. Using Mg⁺², the use of the mutant enzyme provides for high temperature, high fidelity reverse-transcription and amplification of RNA with a usable efficiency.

We Claim:

- 1. A method for reverse transcribing an RNA, that comprises:
- (a) providing a reverse transcription reaction mixture comprising said RNA, a primer, a divalent cation, and a mutant thermoactive DNA polymerase, wherein said mutant DNA polymerase is characterized in that
 - i) in its native form said DNA polymerase comprises an amino acid sequence that is SEQ ID NO: 1;
 - ii) the amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, or P; and
- 10 (b) treating said reaction mixture at a temperature sufficient for said mutant DNA polymerase to initiate synthesis of an extension product of said primer to provide a cDNA molecule complementary to said RNA.
 - 2. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 2.
- 3. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 3.
 - 4. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 4.
- 20 5. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 5.
 - 6. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 6.
- 7. The method of Claim 1, wherein said amino acid sequence is SEQ ID NO: 7.
 - 8. A method of Claim 1, wherein said mutant DNA polymerase is thermostable.
- The method of claim 1, wherein said DNA polymerase is a mutant form of a
 Thermus species DNA polymerase.
 - 10. The method of claim 1, wherein said DNA polymerase is a mutant form of *Thermus thermophilus* DNA polymerase or *Thermus aquaticus* DNA polymerase.

- The method of Claim 1, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
- 12. The method of Claim 1, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, P, Q, or D.
 - 13. A method for reverse transcribing an RNA, that comprises:
- (a) providing a reverse transcription reaction mixture comprising said RNA, a

 primer, Mg⁺², and a mutant thermoactive DNA polymerase, wherein said mutant DNA
 polymerase is characterized in that
 - i) in its native form said DNA polymerase comprises an amino acid sequence that is SEQ ID NO: 1;
 - ii) the amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, or P; and (b) treating said reaction mixture at a temperature sufficient for said mutant DNA polymerase to initiate synthesis of an extension product of said primer to provide a cDNA molecule complementary to said RNA.
- 20 14. The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 2.
 - 15. The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 3.
- The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 4.
 - 17. The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 5.
 - 18. The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 6.
- 30 19. The method of Claim 13, wherein said amino acid sequence is SEQ ID NO: 7.
 - 20. The method of Claim 13, wherein said mutant DNA polymerase is thermostable.

- 21. The method of Claim 13, wherein said DNA polymerase is a mutant form of a *Thermus* species DNA polymerase.
- 22. The method of Claim 13, wherein said DNA polymerase is a mutant form of *Thermus thermophilus* DNA polymerase or *Thermus aquaticus* DNA polymerase.
- 23. The method of Claim 13, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
- 10 24. The method of Claim 13, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, P, Q, or D.
 - 25. A method for amplifying an RNA, that comprise:
- (a) reverse transcribing said RNA according to a method of Claim 1 to provide a cDNA;
 - (b) amplifying said cDNA.
- 26. A method of Claim 25, wherein in step (b) said amplifying is carried out using a polymerase chain reaction.
 - 27. A method for amplifying an RNA, that comprise:
 - (a) reverse transcribing said RNA according to a method of Claim 13 to provide a cDNA;
- 25 (b) amplifying said cDNA.
 - 28. A method of Claim 27, wherein in step (b) said amplifying is carried out using a polymerase chain reaction.

- 29. A method for amplifying an RNA using a single-enzyme reverse transcription/amplification reaction, that comprises:
- (a) providing an amplification reaction mixture comprising said RNA, a pair of primers, a divalent cation, and a mutant thermostable DNA polymerase, wherein said mutant DNA polymerase is characterized in that
 - i) in its native form said DNA polymerase comprises an amino acid sequence that is SEQ ID NO: 1;
 - ii) the amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, or P; and
- (b) treating said reaction mixture at a temperature sufficient for said mutant DNA polymerase to initiate synthesis of an extension product of said primer to provide a cDNA molecule complementary to said RNA;
 - (c) treating said reaction mixture at an appropriate temperature for said mutant DNA polymerase to initiate synthesis of an extension product of said second primer to provide a double-stranded cDNA molecule; and
 - (d) amplifying said double-stranded cDNA molecule of step (c) by a polymerase chain reaction.
- 30. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 2.
 - 31. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 3.
 - 32. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 4.
- 25 33. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 5.
 - 34. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 6.
- 35. The method of Claim 29, wherein said amino acid sequence is SEQ ID NO: 7.
 - 36. The method of Claim 29, wherein said mutant DNA polymerase is thermostable.
 - 37. The method of Claim 29, wherein said DNA polymerase is a mutant form of a *Thermus* species DNA polymerase.

20

- 38. The method of Claim 29, wherein said DNA polymerase is a mutant form of *Thermus thermophilus* DNA polymerase or *Thermus aquaticus* DNA polymerase.
- 5 39. The method of Claim 29, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
- 40. The method of Claim 29, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E,
 10 A, G, P, Q, or D.
 - 41. A method for amplifying an RNA using a single-enzyme reverse transcription/amplification reaction, that comprises:
 - (a) providing an amplification reaction mixture comprising said RNA, a pair of primers, Mg⁺², and a mutant thermostable DNA polymerase, wherein said mutant DNA polymerase is characterized in that
 - i) in its native form said DNA polymerase comprises an amino acid sequence that is SEQ ID NO: 1;
 - ii) the amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, or P; and (b) treating said reaction mixture at a temperature sufficient for said mutant DNA polymerase to initiate synthesis of an extension product of said primer to provide a cDNA molecule complementary to said RNA;
 - (c) treating said reaction mixture at an appropriate temperature for said mutant DNA polymerase to initiate synthesis of an extension product of said second primer to provide a double-stranded cDNA molecule; and
 - (d) amplifying said double-stranded cDNA molecule of step (c) by a polymerase chain reaction.
- 30 42. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 2.
 - 43. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 3.

- 44. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 4.
- 45. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 5.
- 46. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 6.
- 47. The method of Claim 41, wherein said amino acid sequence is SEQ ID NO: 7.
- 10 48. The method of Claim 41, wherein said mutant DNA polymerase is thermostable.
 - 49. The method of Claim 41, wherein said DNA polymerase is a mutant form of a *Thermus* species DNA polymerase.
- 15 50. The method of Claim 41, wherein said DNA polymerase is a mutant form of Thermus thermophilus DNA polymerase or Thermus aquaticus DNA polymerase.
 - 51. The method of Claim 41, wherein said temperature of said reaction mixture in step (b) is between 40°C and 80°C.
 - 52. The method of Claim 41, wherein said amino acid at position 4 of said amino acid sequence is mutated in comparison to said native sequence to an amino acid other than E, A, G, P, Q, or D.

Abstract

The present invention relates to improved reverse transcription methods using a modified thermostable DNA polymerases, particularly in a magnesium ion buffer. These methods are particularly useful in combined reverse-transcription/amplification reactions.

SEQUENCE LISTING

```
<110> Roche Molecular Systems, Inc.
```

<120> HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES

```
<130> RPA1006 Sequence
```

```
<140>
```

<141>

<160> 21

<170> PatentIn Ver. 2.1

<210> 1

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Sequence Motif

<220>

<221> VARIANT

<222> (2)

<223> X is S or A

<220>

<221> VARIANT

<222> (3)

<223> X is any amino acid

<220>

<221> VARIANT

<222> (4)

<223> X is any amino acid

<220>

<221> VARIANT

<222> (6)

<223> X is any amino acid

<220>

<221> VARIANT

<222> (7)

<223> X is any amino acid

```
<220>
<221> VARIANT
<222> (8)
<223> X is any amino acid
<220>
<221> VARIANT
<222> (9)
<223> X is any amino acid
<220>
<221> VARIANT
<222> (9)
<223> X is any amino acid
<220>
<221> VARIANT
<222> (10)
<223> X is any amino acid
<220>
<221> VARIANT
<222> (5)
<223> X is L or I
<400> 1
Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa Glu
1
          5
<210> 2
<211> 11
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Sequence Motif
<220>
<221> VARIANT
<222> (3)
<223> X is Q or G
<220>
<221> VARIANT
<222> (6)
<223> X is S or A
```

```
Leu Ser Xaa Glu Leu Xaa Ile Pro Tyr Glu Glu
                5
  <210> 3
  <211> 11
  <212> PRT
  <213> Artificial Sequence
  <223> Description of Artificial Sequence: Sequence Motif
  <400> 3
  Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
  1 5 , 10
  <210> 4
  <211> 11
  <212> PRT
  <213> Artificial Sequence
  <220>
  <223> Description of Artificial Sequence: Sequence Motif
 <220>
  <221> VARIANT
  <222> (3)
<223> X is Q or G
  <400> 4
. Leu Ser Xaa Glu Leu Ser Ile Pro Tyr Glu Glu
  1 5
  <210> 5
  <211> 11
  <212> PRT
  <213> Artificial Sequence
  <223> Description of Artificial Sequence: Sequence Motif
  <220>
  <221> VARIANT
  <222> (7)
```

```
<223> X is V or I
<400> 5
Leu Ser Val Arg Leu Gly Xaa Pro Val Lys Glu
<210> 6
<211> 11
<212> PRT
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Sequence Motif
<400> 6
Leu Ser Lys Arg Ile Gly Leu Ser Val Ser Glu
 1
<210> 7
<211> 11
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Sequence Motif
<220>
<221> VARIANT
<222> (8)
<223> X is S or T
<400> 7
Leu Ala Gln Asn Leu Asn Ile Xaa Arg Lys Glu
<210> 8
<211> 11
<212> PRT
<213> Thermus aquaticus
<400> 8
Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
```

```
<210> 9 ·
<211> 11
<212> PRT
<213> Thermus flavus
<400> 9
Leu Ser Gly Glu Leu Ser Ile Pro Tyr Glu Glu
                                   10
<210> 10
<211> 11
<212> PRT
<213> Thermus thermophilus
Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
           5
<210> 11
<211> 11
<212> PRT
<213> Thermus sp. 205
<400> 11
Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
                 5
<210> 12
<211> 11
<212> PRT
<213> Thermus sp. sps17
<400> 12
Leu Ser Gln Glu Leu Ser Ile Pro Tyr Glu Glu
1 5
<210> 13
<211> 11
<212> PRT
<213> Thermus caldophilus
<400> 13
Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
                                  10
```

```
<210> 14
<211> 11
<212> PRT
<213> Thermus filiformis
<400> 14
Leu Ser Gln Glu Leu Ser Ile Pro Tyr Glu Glu
               5
<210> 15
<211> 11
<212> PRT
<213> Thermotoga maritima
Leu Ser Val Arg Leu Gly Val Pro Val Lys Glu
       5
<210> 16
<211> 11
<212> PRT
<213> Thermotoga neapolitana
Leu Ser Val Arg Leu Gly Ile Pro Val Lys Glu
1 5
<210> 17
<211> 11
<212> PRT
<213> Thermosipho africanus
<400> 17
Leu Ser Lys Arg Ile Gly Leu Ser Val Ser Glu
<210> 18
<211> 11
<212> PRT
<213> Bacillus caldotenax
<400> 18
```

```
Leu Ala Gln Asn Leu Asn Ile Ser Arg Lys Glu
                5
                                  10
<210> 19
<211> 11
<212> PRT
<213> Bacillus stearothermophilus
Leu Ala Gin Asn Leu Asn Ile Thr Arg Lys Glu
              5
<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Primer
<400> 20
cgagatccct ccaaaatcaa
                                                                 20
<210> 21
<211> 23
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Primer
<400> 21
                                                                 23
catgagtcct tccacgatac caa
```

Declaration and Power of Attorney for Patent Application

As the below named inventors, We hereby declare that:

Our residences, post office addresses, and citizenships are as stated below our names,

We believe we are the original, first and sole inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

HIGH TEMPERATURE REVERSE TRANSCRIPTION USING MUTANT DNA POLYMERASES

as	
contents of the above-	identified specification, including the
rial to patentability as	defined in 37 CFR §1.56.
lication which designat by checking the box,	any foreign application(s) for patent or ted at least one country other than the any foreign application for patent or of the application on which priority is
	Priority Claimed
Month/Year Filed)	Priority Claimed [] [] Yes No
֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜֜	contents of the above- erial to patentability as of 9(a)-(d) or \$365(b) of a lication which designate by checking the box,

	(Filing Date)	(Application No.)	(Filing Date)
(Application No.)	(Filing Date)	(Application No.)	(Filing Date)
<i>t.</i>		:	
claims of this application is of Title 35, United States (s not disclosed in the prior Un Code, §112, I acknowledge the which became available between	ates, listed below and, insofar as to ited States application in the manner are duty to disclose information who seen the filing date of the prior app	er provided by the first paragraphich is material to patentability
i	(Pill - Day)	(600.0)	
? (Application Serial No.))	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	******* d- b:6	un knowledge are true and that all	
I hereby declare that all state and belief are believed to statements and the like so must be code and that such we have the states code and the	be true; and further that the	nese statements were made with to imprisonment, or both, under Section opardize the validity of the applications.	he knowledge that willful fals
I hereby declare that all state and belief are believed to statements and the like so must be code and that such we power of ATTORNEY	be true; and further that the nade are punishable by fine or willful false statements may jet ': As named inventors, we	ese statements were made with t	he knowledge that willful fals on 1001 of Title 18 of the Unite ion or any patent issued thereon at Customer Number 22829 t

EDWARD SOH SMITH	· ·
Full name of sole or first inventor	
EN 46mith	4/17/00 Date
Inventor's signature	Date
San Francisco, CA	
Residence	
	-
US	
Citizenship	
2379 Bryant Street, San Francisco, CA 94110	
Post Office Address	
	•
() CARITA MARIA ELECTROM	
CARITA MARIA ELFSTROM Full name of second joint inventor	
·	
al hall	4117/00
[]Inventor's signature	Date
(I)	
San Francisco, CA	
-≛US	
Citizenship	
5285 Diamond Heights Boulevard, Apt. #103, San Francisco, Post Office Address	CA 94131
rost Office Address	1
DAVID HARROW GELFAND	
Full name of second joint inventor	
Janil M. Sola J	4-14-00
Inventor's signature	Date
Oakland, CA	
Residence	
US	
Citizenship	
•	•
6208 Chelton Drive, Oakland, CA 94611	
Post Office Address	

RUSSELL GENE HIGUCHI		
Full name of second joint inventor		 'ı
Purs Il Com / Hym 1'	Date 4/17/00	
Alameda, CA		
Residence		
	<u> </u>	
US Citizenship		
Ciuzensnip		
3258 Liberty Avenue, Alameda, CA 94501		
Post Office Address		
.~	. *	
	;	
THOMAS WILLIAM MYERS	_	
Full name of second joint inventor		
11 / Amerila. Il mono	04-14-2000	
i Inventor's signature	Date	
lı¦ çi}Alameda, CA		
Residence		
#US		
#Citizenship		
MONO Femelika Baulannak Alamaka GA 04601		
2910 Fernside Boulevard, Alameda, CA 94501 Post Office Address		
1 501 511100 11011003	,	
	•	
NANCY JENEANE SCHÖNBRUNNER Full name of second joint inventor		
r dit name of second joint inventor		
	11 17 00	
grang f. an	4.17.00	
Inventor's signature	Date	
Moraga, CA		
Residence		
US		
Citizenship		
14 Wandal Drive Morroy C4 04555		
14 Wandel Drive, Moraga, CA 94556 Post Office Address		

gn	
ū	
ü	
W	
IJ	
Ü	
C	
=	
卢스	
Ø	
Ö	
~~	

ALICE MING WANG	
Full name of second joint inventor	
Ali min /	4/14/00
Inventor's signature	Date
Lafayette, CA	
Residence .	
	;
US	•
Citizenship	
	•
1246 Quandt Road, Lafayette, CA 94549	
Post Office Address	

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.